CADMIUM TOXICITY AND CADMIUM RESISTANCE IN YEAST

A study of possible mechanisms

B.G.F. Kessels

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

In its pure form cadmium is a silvery white and relatively soft metal. Together with for example mercury, lead, cobalt, copper, nickel and zinc, cadmium forms part of the group of heavy metals, which comprises 40 elements in total. In all stable chemical compounds, cadmium occurs as a divalent ion. Cadmium is a relatively rare element with no known biological function (Wood, 1974, Babich and Stotzky, 1978). It is normally found in the natural environment at low levels (Iverson and Brinckman, 1978) and occurs mainly as a component of minerals in the earth's crust. Cadmium can enter the environment from various sources, such as by-products from zinc refining, coal combustion, mine wastes, electroplating processes, nickel-cadmium batteries, iron and steel production, fertilizers, pesticides and paint pigments (Hutton, 1983, Iverson and Brinckman, 1978). Industrial utilization of cadmium has accelerated the rates of biological mobilization and transport of this element, as the input by industry far exceeds the amounts involved in natural abiotic cycling processes (Babich and Stotzky, 1978).

As a result of the rapidly increasing cadmium content of the environment, organisms are more and more exposed to this toxic metal. In plants cadmium causes decreased growth (John and Van Laerhoven, 1976) and carbohydrate metabolism is affected (Lee et al., 1975). In vitro studies with mitochondria from Zea mays L. showed that cadmium inhibits succinate oxidation (Miller et al., 1973). Most plants are able to synthesize small sulfur-rich proteins that bind heavy metals. Also in the yeast Schizosaccharomyces pombe these so called phytochelatins were demonstrated (Grill et al., 1986). The function of phytochelatins probably is to bind heavy metal ions and thus to prevent the toxic action of these metal ions. In plants, resistance against the toxic action of heavy metals may very well be correlated with synthesis of phytochelatins.

Cadmium provokes a variety of biological responses in both human beings and animals. Food constitutes the principal environmental source of cadmium exposure to the non-smoking human population and estimates of daily intake range between 20 and 70 µg/day (Travis and Etnier, 1982). Because specific mechanisms for the toxic action of cadmium are as yet unidentified, the long biological half-life (15-30 years) and the continuous human exposure

via the food chain are convincing arguments to justify thorough investigation into the interaction of cadmium with the living cell. The greatest accumulation occurs in the liver and kidney whereas the critical target organs are the liver, kidney and testes (Samarawickrama, 1979). The liver plays a key role in the disposition of both cadmium and zinc This is not only due to the inducibility of a metal binding protein known as metallothionein but also to the rapid uptake of these metals into the liver. This rapid uptake probably is caused by a metabolism dependent carrier mediated transport. In addition passive diffusion contributes to the uptake (Gerson and Shaikh, 1984). Malfunction of the kidneys is often the first detectable effect of excessive exposure of mammalians to heavy metals. Especially the renal cortex is used to detect intoxication by heavy metals. Recently whole body X-ray diffraction spectroscopy was introduced as a method for detecting the accumulation of heavy metals in mammalian kidneys without application of surgery. Heavy metal intoxication of mammalians also can lead to hypertension, affection of the nerve system, emphysema, testicular necrosis and development of many forms of cancer (Prasada Rao et al., 1983).

Cadmium interaction with cellular components results from the formation of either ionic or covalent complexes with electron donor atoms present in compounds containing sulphur, nitrogen and oxigen (Webb, 1979). As a result cadmium interacts with many cellular constituents including proteins, phospholipids and nucleic acids. One of the most important aspects in Cd toxicity seems to be the interaction of cadmium with sulfhydryl (SH) groups. It has been suggested by several authors that the toxicity of heavy metals in general is based upon their interaction with SH groups (Rothstein, 1959, Vallee and Ulmer, 1972, Chvapil, 1973, Webb, 1979, Christie and Costa, 1984).

Cadmium is also extremely toxic to microorganisms such as bacteria, algae and fungi. Nevertheless, there are microbial strains that are resistant to cadmium. Cd resistance in microorganisms in general comprises a reduced cadmium uptake and not enzymic transformation of the metal into a non-toxic form. Recently the genetic basis for cadmium resistance, the mechanisms of cadmium uptake and the mechanisms of cadmium toxicity became active areas of research in microbiology (Trevors et al., 1986). There are several reasons for studying the way cadmium interferes with the living

cells by using microorganisms under laboratory conditions. The genetical constitution of microorganisms is less complex and better understood than the genetical constitution of higher organisms. Furthermore microorganisms can easily be grown in defined media under precisely controlled conditions and at relatively low costs. Microorganisms grow quickly, are continuously available and appropriate results can be obtained on short terms. Together with other advantages, unicellular microorganisms render a unique possibility for the study of cadmium toxicity and cadmium resistance. In the natural environment (soil, sludge water and sediment) Cd toxicity in microorganisms is influenced by numerous interacting factors. This should be kept in mind during research conducted in synthetic media or in altered (disturbed and (or) sterilized) environmental samples (Babich and Stotzky, 1978).

Surowitz et al. (1984) examined the effects of Cd^{2+} accumulation on the growth and respiration of both a Cd sensitive and a Cd resistant of the bacterium Bacillus subtilis. Growth and respiration of the sensitive strain were significantly inhibited by 90 µM Cd^{2+}, while the resistant strain was only slightly affected. Protoplasts of the tolerant strain were even more resistant to Cd^{2+} than the whole cells from which they were obtained from. It appeared that the difference in resistance between the two strains of B. subtilis was due to a reduced Cd^{2+} accumulation in the resistant strain. After four h of exposure to 9 µM Cd^{2+}, the Cd sensitive strain had accumulated about 10-fold more Cd^{2+} than the resistant strain (Surowitz et al., 1984). In addition, from the inhibitory effect of cyanide upon accumulation of Cd^{2+} by protoplasts obtained from the cadmium sensitive strain it was concluded that the uptake of Cd^{2+} by this strain was an active process, whereas Cd^{2+} uptake by the resistant strain appeared to be a passive process (Laddaga et al., 1985). By the same authors indications were found for accumulation of Cd^{2+} by means of the active Mn^{2+}-transport system in cadmium sensitive cells of B. subtilis. The cadmium resistant strain, however, showed a reduced Cd^{2+} accumulation though Mn^{2+} transport was not influenced (Laddaga et al., 1985).

In Staphylococcus aureus 17810R, a plasmid controlled reduced uptake of Cd^{2+} has been shown (Tynecka et al., 1981). In fact an energy-dependent efflux system prevents loading of the cells with

Cd^{2+}. Efflux of Cd^{2+} from this strain may proceed by means of an antiporter that exchanges cellular Cd^{2+} for protons of the medium. Silver and Misra, 1984 identified the gene responsible for the high resistance against Cd^{2+} in 1780R (gene cadA). At the same time the authors reported about a second gene (cadB) encoding for a small increase in cadmium resistance, which also is located on a plasmid. The mechanism by which this cadB gene mediates the low level of cadmium resistance is still unknown (Tynecka et al., 1981).

The toxicity of cadmium to fungi and other microbial groups can be influenced by the physical and chemical nature of the natural growth environment or the artificial nutrient medium. For example the pH of an environment affects the metal-microbe interactions and toxicity may be increased or decreased. The toxicity of Cd^{2+} towards certain fungi was increased on increasing the pH values to 8-9, whereas the toxic effects of cadmium upon yeast were maximal at low pH values (Trevors et al., 1986). Other anions and cations also can affect the toxicity of cadmium. In many studies calcium appeared to possess a protective effect upon cadmium intoxication (Norris and Kelly, 1977, Kessels et al., 1985). Anions such as phosphate, sulphide and carbonate also can reduce cadmium toxicity by the formation of insoluble precipitates. Furthermore organic compounds, e.g. amino acids, proteins, polysaccharides and chelating agents present in the medium may reduce the toxicity of cadmium by binding or complexation although in some cases the metal complexes may be more toxic than the free ions (Gadd and Griffiths, 1978).

Yeasts exhibit a variety of species- and strain-specific responses to cadmium. Itoh et al., 1975, found that Cd^{2+} was more toxic to Saccharomyces cerevisiae than were the ions of Ag, Hg, Cu and Ni. As mentioned before, many factors may alter the toxicity of cadmium towards microorganisms. Comparison of sensitivities towards cadmium reported in literature therefore is almost impossible. Studies on cadmium toxicity in yeasts often comprise comparison of wild type strains with strains displaying a decreased sensitivity towards cadmium. There are two ways by which yeast strains may acquire a decreased sensitivity for cadmium. One way is by means of physiological adaptation When a strain is repeatedly subcultured in cadmium containing media, physiological adaptation may render a decreased sensitivity towards the metal. When such a

cadmium adapted yeast strain is transferred to a cadmium free nutrient medium several times, the sensitivity towards cadmium increases again. The second way by which yeast cells may acquire a decreased sensitivity towards cadmium is by mutation and selection. Such a cadmium resistant strain also may be obtained by repeated subculturing in cadmium containing nutrient media. However, a cadmium resistant strain does not loose its decreased sensitivity towards cadmium during subculturing in cadmium-free media. Mostly, cadmium adapted yeast strains can withstand higher cadmium concentrations in the nutrient medium than cadmium resistant yeast strains (Trevors et al., 1986).

The preceeding section shows that a large body of information concerning the toxicity of cadmium in living cells has become available during the last years. Nevertheless, there is still little known about the mechanism by which this heavy metal exerts its toxic effects in yeast. The study described in this thesis was therefore aimed to gain more insight into the mechanism by which Cd^{2+} intoxicates the yeast cells and in addition into the mechanism by which yeast cells may acquire resistance against the intoxication by cadmium.

We first examined the way by which cadmium may interact with the yeast cells. In chapter 2 we examined the effect of Cd^{2+} upon cell growth of S. cerevisiae. It was further examined wether the inhibitory effect of Cd^{2+} upon cell growth could be reduced by the divalent cation Ca^{2+} as a protective agent. Cd^{2+} and Ca^{2+} resemble each other quite well having approximately the same ionic radius. Therefore it might be possible that the non-toxic Ca^{2+} will protect the yeast cells against the toxic action of Cd^{2+}. Both cations may compete for the same binding groups involved in the intoxication process. We also examined wether efflux of K^{+} which accompanies intoxication of the yeast cells by Cd^{2+} (Norris and Kelly, 1977, Gadd and Mowll, 1983) is also affected by Ca^{2+}. Furthermore it was investigated wether Cd^{2+} acts at the outer side of the yeast cell membrane or at the inner side.

A large number of organic compounds that are toxic to yeast cells stimulate the uptake of Ca^{2+} and Sr^{2+} by the cells with concomitant release of cell K^{+}. This applies to the

inhibitors of the plasmamembrane ATPase DIO-9 (Foury et al, 1977, Borst-Pauwels et al., 1986a) miconazole (Borst-Pauwels et al., 1986a), diethylstilbestrol (Borst-Pauwels et al., 1984), ethidium (Borst-Pauwels et al., 1986b, Peña, 1978, Theuvenet et al., 1986) and suloctidil (Dufour et al., 1980), the calmodulin antagonists trifluoperazin (Borst-pauwels et al., 1986b, Eilam, 1983, 1984), compound 48/80 and calmidazolium (Borst-Pauwels et al., 1986b) and chlorpromazin (Eilam, 1984) which probably also act by virtue of their inhibitory action on the membrane ATPase, the polyene antibiotic nystatin (Johnson et al., 1978) and the membrane permeabilizing agents DEAE-dextran (Theuvenet et al., 1986) and chitosan (Borst-Pauwels et al, 1986a), as well. As far as examined, these compounds also increase the influx of the lipophylic cation tetraphenylphosphonium (TPP) which is used in yeast as an indicator for the membrane potential (Eilam, 1983, 1984, Borst-Pauwels et al., 1986a). We were now anxious to know wether also Cd^{2+} exerts a stimulatory effect upon Ca^{2+} and TPP uptake in yeast under conditions that the heavy metal provokes K^{+} release. The results are described in chapter 3.

In chapter 4-9 we compared the properties of wild type cells and cadmium resistant cells. Hayashi et al., 1986 and Joho et al., 1986, developed cadmium resistant strains of both S. cerevisiae and S. pombe. According to their findings cadmium resistance comprises both a reduced uptake of cadmium and synthesis of cadmium binding proteins in the yeast strains examined. Wild type strains of S. cerevisiae and of S. pombe together with cadmium resistant strains of both yeasts were used in that study. In chapter 4 we tried to support this finding that resistance of yeast against cadmium is partly due to the formation of cadmium binding proteins which may bind intracellular cadmium and thus give rise to a decrease in free cytosolic Cd^{2+}. We compared wild type cells with cadmium resistant cells of both S. cerevisiae and S. pombe.

Other candidates for trapping intracellularly accumulated Cd^{2+} are the polyphosphates . Besides some localisation in the periplasmatic space of the yeast cells (Thijsen et al., 1981), polyphosphates are mainly localized in the vacuoles (Urech et al., 1978) and are able to bind polyvalent cations(Durr et al., 1979). Therefore they also may play a role in the mechanism of cadmium detoxification of the yeast cells. We have now examined in chapter 5

whether resistance against cadmium was accompanied by an increase in the polyphosphate content of the cell. The polyphosphates are determined both by chemical methods and by ^{31}P NMR. The latter method was compared with the first one in order to allow us to draw conclusions concerning the state of the polyphosphates present. Complexation of the polyphosphates namely may give rise to broadening of the ^{31}P signal and thereby complexed polyphosphates remain unobserved in the ^{31}P spectra whereas in the chemical method all polyphosphates being present are determined.

The organic compounds which give rise to a concomitant increase in K^+ efflux and Ca^{2+} or Sr^{2+} uptake share the property of being inhibitors of the plasmamembrane ATPase, which fuctions as a proton pump. They also cause a larger loss of cell K^+ from metabolizing cells than from non-metabolizing cells. As will be shown in chapter 3 Cd^{2+} gives rise to concomitant loss of cell K^+ and increased uptake of Ca^{2+}, as well and the Cd^{2+} provoked K^+ efflux is greatly increased by metabolism. We therefore considered the possibility that inhibition of the membrane ATPase contributes to the toxic effect of cadmium in yeast and that resistance against Cd^{2+} is due to a reduced sensitivity of the ATPase towards cadmium. In chapter 6 we studied the cadmium sensitivity of the ATPase in isolated plasmamembranes of both wild type and cadmium resistant cells of S. cerevisiae.

In chapter 7 a study analogue to that of chapter 2 is described but now the experiments were performed with cadmium resistant cells of S. cerevisiae. We studied the effect of cadmium upon K^+ efflux from cadmium resistant cells and compared the results with those obtained with wild type cells. Furthermore we examined wether uptake of Cd^{2+} into cadmium resistant cells was reduced as has been shown to be the case in a cadmium resistant strain of S. cerevisiae isolated by Joho, 1985.

As already has been stated, various organic xenobiotics have a similar effect as Cd^{2+} upon yeast cells. They also give rise to K^+ efflux from yeast cells. As shown in chapter 3, the similarities between these xenobiotics and Cd^{2+} also refer to the concomitant increase in the influx of calcium and the uptake of TTP. We have now examined whether cells which were rendered resistant against cadmium also were less sensitive towards these xenobiotics. We therefore examined in chapter 8 the effect of

several xenobiotics upon K^+ efflux from both wild type and cadmium resistant cells of S. cerevisiae. In order to be sure that an eventual difference in K^+ efflux was indeed due to changes in sensitivity towards cadmium, we also examined the effect of the xenobiotics upon cell growth.

In chapter 9 we compared the effect of cadmium upon K^+ efflux from both wild type and cadmium resistant cells and upon the viability of these cells. The aim of this study was to examine whether efflux of K^+ was indeed a good measure for the intoxication of the yeast cells by cadmium. During the preparation of this thesis accumulating evidence appeared from studies on the effect of organic xenobiotics upon yeast, that these xenobiotics interact with the cells by means of an all-or-none process (e.g. Borst-Pauwels and Theuvenet, 1985). Part of the cells become damaged during the interaction with these compounds, giving rise to an almost complete K^+ loss, whereas the remainder part of the cells in the suspension still remains intact. During the intoxication process the fraction of damaged cells increases gradually. As long as part of the cells are still intact K^+ released from the damaged cells will be taken up by the still intact cells. That means that efflux of K^+ is no good quantitative measure for the intoxication process. We have now examined wether cadmium may also interfere with yeast cells according to an all-or-none process. If this is true part of the cells may loose their viability while the remainder may still be viable. This was studied by applying the spread plate technique to both wild type and cadmium resistant cells and on comparing these results with those obtained from the K^+ release experiments.

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CHAPTER 2

PROTECTION OF SACCHAROMYCES CEREVISIAE AGAINST Cd^{2+} TOXICITY BY Ca^{2+}

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Protection of *Saccharomyces cerevisiae* against Cd^{2+} Toxicity by Ca^{2+}

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Ca^{2+} protected yeast cells very effectively against the toxic effects of Cd^{2+}; Mg^{2+} had only a slight protecting effect as far as protection against Cd^{2+}-induced release of K^+ was concerned. Protection of the yeast cells against Cd^{2+} toxicity was due to a reduction in Cd^{2+} uptake in the presence of Ca^{2+}. A single relationship existed between the relative rate of K^+ release induced by Cd^{2+} and the cellular Cd^{2+} concentration. Within the first few minutes of incubating cells with Cd^{2+}, the molar ratio of K^+ released and Cd^{2+} accumulated was 22 and was independent of the amount of $CdCl_2$ added. This ratio decreased during incubation of the cells with Cd^{2+}, depending on the external Cd^{2+} concentration.

INTRODUCTION

Yeast growth can be inhibited by Cd^{2+} (Heldwein *et al.*, 1977; Macara, 1978; Bitton *et al.*, 1984). Like Ag^{2+} and Hg^{2+}, Cd^{2+} exerts this toxic effect at relatively low concentrations (Grafl & Schwantes, 1983). Besides inhibition of yeast growth, Cd^{2+} also induces the release of K^+ from the cells. This release of K^+ is larger than can be accounted for by an electroneutral 2:1 exchange with Cd^{2+} (Norris & Kelly, 1977; Gadd & Mowll, 1983). Apparently Cd^{2+} increases the permeability of the plasmalemma for K^+.

The rate of Cd^{2+} uptake by yeast can be reduced by the addition of Ca^{2+} to the yeast suspension (Norris & Kelly, 1977). Cd^{2+}-induced release of K^+ from these cells also is diminished. With Mg^{2+} on the other hand, only a small reduction in Cd^{2+} uptake occurs (Norris & Kelly, 1977).

This paper examines whether Ca^{2+} also protects against growth inhibition of yeast caused by Cd^{2+}, and in addition determines more closely the dependence of Cd^{2+}-induced K^+ release on the cellular Cd^{2+} concentration.

METHODS

The diploid strain Delft II of *Saccharomyces cerevisiae* was grown in medium A [1% (w/v) Yeast Extract, 2% (w/v) Bacto-Peptone, 2% (w/v) glucose, 0·2% (w/v) $MgCl_2$ $6H_2O$, 3·46% (w/v) KH_2PO_4, 0·04% (w/v) K_2HPO_4, brought to pH 4·5 with HCl] on an orbital shaker at 125 r.p.m. in 250 ml batch cultures at 30 °C. For growth experiments 250 ml medium A was inoculated with 0·5 ml from a stationary phase culture. Samples, taken at appropriate times during growth and ranging from 5 to 20 ml in volume, were centrifuged, washed twice with 1 ml distilled water, resuspended in 1 ml distilled water and dried to constant weight at 105 °C in aluminium trays.

In some cases defined Yeast Nitrogen Base (YNB) medium was used for growth experiments and growth was determined with a haemocytometer (Burke). Viability was determined by the spread plate technique using sterile distilled water as diluent.

For Cd^{2+} uptake and K^+ release experiments, stationary phase cells were harvested by centrifugation, washed twice with 125 ml distilled water and resuspended in 45 mM-Tris/succinate buffer at pH 5·0. Cells were pre-incubated for 30 min in the presence of 3% (w/v) glucose at 25 °C under anaerobic conditions and $CdCl_2$ was added at appropriate concentrations. For determination of Cd^{2+} uptake tracer amounts of $^{109}CdCl_2$ (10^5 c.p.m. ml^{-1}) were added together with the non-radioactive $CdCl_2$. The radioactivity in samples taken according to the procedure of (Roomans *et al.*, 1979) was measured in a liquid scintillation analyser (Philips). K^+

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release during $CdCl_2$ incubation was determined by flame spectrophotometry according to Kuypers & Roomans (1979) Cd^{2+} activities were measured with a cadmium selectrode type F 3003 (Radiometer Kopenhagen)

$^{109}CdCl_2$ [specific activity 158 7 Ci g^{-1} (5872 GBq g^{-1})] was purchased from Amersham Yeast Extract Bacto-Peptone Yeast Nitrogen Base and Bacto-Agar were purchased from Difco All other chemicals were reagent grade and obtained from commercial sources

RESULTS

Growth inhibition by Cd^{2+} and the protective effect of Ca^{2+}

Medium A, used for growth of the yeast, is rich in phosphate, protein and other cadmium-binding constituents Therefore the free Cd^{2+} concentration in medium A was always much lower than the total $CdCl_2$ concentration administered Furthermore, heat sterilization of medium A already containing $CdCl_2$ resulted in Cd-containing precipitates at all $CdCl_2$ concentrations applied However, when $CdCl_2$ was administered from a sterile stock solution to sterile medium A, precipitates were only formed when the final $CdCl_2$ concentration exceeded 2 mM For this reason we used the latter method with concentrations of $CdCl_2$ not exceeding 1 mM

In order to estimate the extent of Cd^{2+} complexation Cd^{2+} activities were measured in several media with a cadmium selectrode For all $CdCl_2$ concentrations tested, the Cd^{2+} activity in medium A was about 10% of the corresponding values obtained in distilled water For 45 mM-Tris/succinate buffer about 58% of the Cd^{2+} activity determined in distilled water was obtained and for YNB medium about 90%

Both 0 1 mM $CdCl_2$ and 1 mM-$CdCl_2$ inhibited growth of the yeast cells in medium A (Fig 1*a*) After addition of 0 1 mM-$CdCl_2$ growth was retarded severely and after 8 h incubation growth almost stopped With 1 mM-$CdCl_2$ present in the medium, growth stopped immediately, followed by a slight transient recovery 4 h after Cd^{2+} administration After 20 h with 1 mM-$CdCl_2$ in the medium the feeble growth changed to a decrease in dry weight yield

Ca^{2+} (1 mM) partly protected the cells against Cd^{2+} toxicity (Fig 1*b*) With both 0 1 and 1 mM-$CdCl_2$ growth was much less retarded than in the absence of Ca^{2+} At 0 1 mM-Cd^{2+} growth inhibition was not immediately maximal while after addition of 1 mM-$CdCl_2$ growth ceased almost immediately followed by a partial recovery

The protective effect of Ca^{2+} on Cd^{2+}-induced growth inhibition was also studied in the defined YNB medium After 6 h growth ($1\ 3 \times 10^6$ cells ml^{-1}) Cd^{2+} with or without Ca^{2+} was added The cell concentrations 30 h later were $4\ 0 \pm 0\ 3$, $0\ 37 \pm 0\ 03$, $0\ 94 \pm 0\ 07$ and $4\ 4 \pm 0\ 3 \times 10^7$ cells ml^{-1} for the control, 0 1 mM-$CdCl_2$, 0 1 mM-$CdCl_2$ plus 1 mM-$CaCl_2$ and 1 mM-$CaCl_2$ respectively During these 30 h incubation with 0 1 mM-$CdCl_2$, the viability of the cells dropped to about 16% of the viability of the control without $CdCl_2$

Release of cell K^+ during Cd^{2+} uptake and the effects of Ca^{2+} or Mg^{2+}

Besides the growth inhibition experiments the effect of Cd^{2+} on K^+ release from the yeast cells was also examined For these experiments the cells were suspended in 45 mM-Tris/succinate at pH 5 0 This medium is virtually K^+-free Furthermore, binding of Cd^{2+} to constituents of the medium was much less than in the growth medium A

In cells in which metabolism was greatly reduced by the addition of 2-deoxyglucose and antimycin A no detectable Cd^{2+} uptake occurred (Fig 2*a*) and Cd^{2+}-induced K^+ release was very low (Fig 2*b*) On the other hand, with metabolizing cells and in the absence of added Mg^{2+} or Ca^{2+} almost 100% of the cellular K^+ was lost within 2 h of the addition of 1 mM-$CdCl_2$, whereas Cd^{2+} accumulated in the cells to a much higher extent than in the non-metabolizing cells Fig 2 also shows that addition of 1 mM-$CaCl_2$ drastically reduced both Cd^{2+} uptake and Cd^{2+}-induced K^+ release On the other hand, 1 mM-$MgCl_2$ had only a slight effect on Cd^{2+} uptake and K^+ release The K^+ efflux induced by Cd^{2+} both in the absence and in the presence of 1 mM-$MgCl_2$ increased more than proportionally with time during the first 20 min after the addition of 1 mM-$CdCl_2$ Apparently the K^+ efflux rate was not immediately maximal and increased initially (Fig 3*b*)

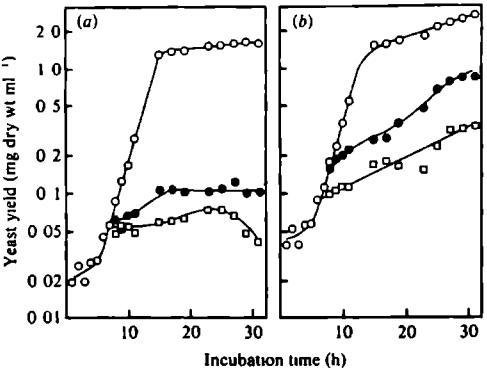

Fig 1 Effect of $CdCl_2$ on yeast growth in the absence (*a*) and presence (*b*) of 1 mM-$CaCl_2$ ○, no $CdCl_2$, ●, 0 1 mM-$CdCl_2$, □, 1 mM-$CdCl_2$ In all cases $CdCl_2$ was added 7 h after inoculation

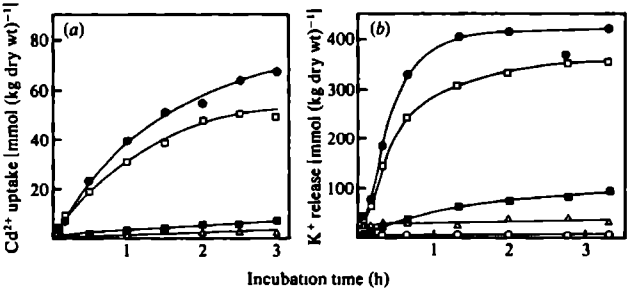

Fig 2 Effect of Ca^{2+}, Mg^{2+} and inhibition of metabolism on Cd^{2+} uptake (*a*) and K^+ release (*b*) of yeast cells ○, no $CdCl_2$, ●, △, 1 mM-$CdCl_2$, □, 1 mM-$CdCl_2$ plus 1 mM-$MgCl_2$, ■, 1 mM-$CdCl_2$ plus 1 mM-$CaCl_2$, ○, ●, □, ■, cells were pre-incubated with 3% (w/v) glucose, △, cells were pre-incubated with 5 mM-2-deoxyglucose and 15 μM-antimycin A The total K^+ content of the cells was 404 mmol (kg dry wt)$^{-1}$

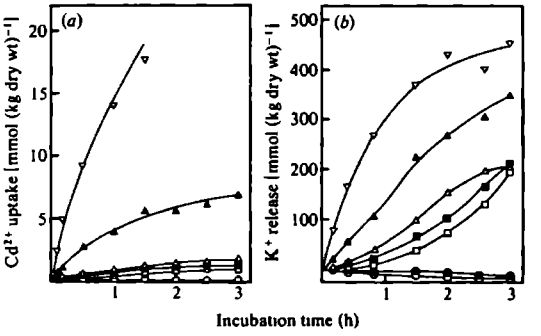

Fig 3 Dependence of Cd^{2+} uptake (*a*) and K^+ release (*b*) on external $CdCl_2$ concentrations ○, no $CdCl_2$, ● 1 μM-$CdCl_2$, □, 5 μM-$CdCl_2$, ■, 10 μM-$CdCl_2$, △, 20 μM-$CdCl_2$, ▲, 100 μM-$CdCl_2$, ▽, 500 μM-$CdCl_2$ The cells were pre-incubated with 3% (w/v) glucose and their total K^+ content was 476 mmol (kg dry wt)$^{-1}$

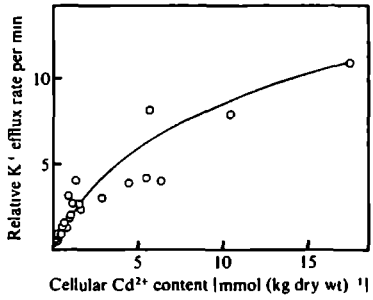

Fig 4 Relation between relative rate of K^+ efflux and cellular Cd^{2+} content during incubation in the presence of 5 500 µM-$CdCl_2$ in the cell suspension (taken from the results shown in Fig 3)

Dependence of Cd^{2+} uptake and K^+ release on Cd^{2+} concentration in the medium

With increasing Cd^{2+} concentration in the Tris/succinate medium the rate of Cd^{2+} uptake by the yeast cells also increased (Fig 3*a*) Concomitantly, K^+ release increased with increasing Cd^{2+} concentration in the medium (Fig 3*b*) The rate of K^+ efflux increased initially during Cd^{2+} uptake with $CdCl_2$ concentrations in the medium ranging from 5 to 100 µM A sigmoidal dependence of K^+ release on incubation time was seen with 20 and 100 µM-$CdCl_2$ In the absence of Cd^{2+} and at 1 µM-Cd^{2+}, however, K^+ was taken up

Norris & Kelly (1977) and Gadd & Mowll (1983) reported that during Cd^{2+} uptake by yeast, K^+ release is much higher than expected for a 2 1 stoicheiometry between K^+ release and Cd^{2+} uptake Our results confirm this A linear relationship existed between K^+ release and Cd^{2+} uptake during the first 5 min of incubation with the different $CdCl_2$ concentrations The number of moles of K^+ released within the first 5 min after Cd^{2+} addition appeared to be about 22 times higher than the equivalent amount of Cd^{2+} absorbed in the cells during the same period, independently of the external Cd^{2+} concentration (up to 0 5 mM) However, no single relation existed between the amount of K^+ released and the cellular Cd^{2+} concentration during longer periods of incubation of the cells with $CdCl_2$ In fact, the ratio of the amount of K^+ released to the cellular Cd^{2+} concentration decreased during incubation of the cells with $CdCl_2$ This decrease became stronger on increasing the external $CdCl_2$ concentration

When the relative K^+ efflux rate, expressed as rate of K^+ efflux [mmol min^{-1} (kg dry wt)$^{-1}$], divided by the residual cellular K^+ content [mmol (kg dry wt)$^{-1}$] is plotted against the cellular Cd^{2+} content [mmol (kg dry wt)$^{-1}$], again there is a linear relationship for low internal Cd^{2+} concentrations For higher cellular Cd^{2+} concentrations the increase in the relative K^+ efflux rate is smaller than the accompanying increase in the cellular Cd^{2+} content (Fig 4)

DISCUSSION

Our results show that Ca^{2+} decreases not only the Cd^{2+}-induced K^+ release (Norris & Kelly, 1977) but also the toxic effect of Cd^{2+} on yeast growth On the other hand, yeast growth with 9 8 mM-Mg^{2+} present in the growth medium is still greatly decreased by the addition of 0 1 or 1 mM-$CdCl_2$ and the Cd^{2+}-induced K^+ release is much less decreased by 1 mM-Mg^{2+} than by 1 mM-Ca^{2+} The greater effectiveness of Ca^{2+} in protecting the cells against Cd^{2+} toxicity is probably due to the fact that Ca^{2+} reduces Cd^{2+} uptake by the cells much more than Mg^{2+} does (Norris & Kelly, 1977) Apparently Cd^{2+} exerts its toxic effect inside the cell This view is in accordance with the minimal K^+ release and Cd^{2+} uptake seen with non-metabolizing cells, when compared to the relatively high K^+ release and Cd^{2+} uptake with metabolizing cells Therefore our results support the findings of Norris & Kelly (1977), Gadd & Mowll (1983), Heldwein *et al* (1977) and Macara (1978)

Norris & Kelly (1977) have shown that for each Cd^{2+} ion accumulated in the yeast cells approximately 4 K^+ ions are released Apparently about twice as much K^+ is released than is required for maintenance of the electroneutrality of the yeast cells We have now found that as many as 22 K^+ ions per Cd^{2+} ion accumulated in the cells are released during the first few minutes after $CdCl_2$ addition to the yeast suspension The discrepancy between values found by us and those of Norris & Kelly (1977) can readily be explained since the ratio of K^+ released to Cd^{2+} accumulated strongly depends upon the duration of Cd^{2+} incubation As a matter of fact this ratio decreased during incubation of the cells with $CdCl_2$ and the decrease became stronger with increasing $CdCl_2$ concentrations in the medium The dependence of the ratio between K^+ released and Cd^{2+} accumulated on the time of incubation is a consequence of the fact that the rate of K^+ release rather than the amount of K^+ released is directly related to the cellular Cd^{2+} concentration The results shown in Fig 4 indicate that saturable sites are involved in the interaction of Cd^{2+} with the yeast cells, since the dependence of the relative rate of K^+ release on the cellular Cd^{2+} concentration shows saturation kinetics The cellular Cd^{2+} concentration at which half-maximal stimulation of K^+ release was found is 4 mM Since probably a large part of the Cd^{2+} is bound to yeast cell constituents, the concentration of free Cd^{2+} at which half-maximal stimulation occurs will be lower

As to the mechanism of Cd^{2+} toxicity in yeast cells, we can only guess at this stage The enhancement of K^+ efflux is a typical phenomenon upon addition of various kinds of toxic agents K^+ release occurs with heavy metals like mercury and copper (Passow & Rothstein, 1960, Shieh & Barber, 1973, Kuyper & Roomans, 1979) and various organic cations (Peña & Ramírez, 1975 Dufour *et al*, 1980, Borst-Pauwels *et al*, 1983, Eilam, 1983) As far as they have been investigated, these agents appear to be inhibitors of the yeast plasma membrane ATPase This is also true for Cd^{2+} (data not shown) Whether the induction of K^+ release from the yeast is a direct effect of an impairment of the membrane ATPase or only an indirect effect is now the subject of further examination

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CHAPTER 3

CHANGES IN ^{45}Ca AND ^{109}Cd UPTAKE, MEMBRANE POTENTIAL AND CELL pH IN SACCHAROMYCES CEREVISIAE PROVOKED BY Cd^{2+}

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Changes in ^{45}Ca and ^{109}Cd Uptake, Membrane Potential and Cell pH in *Saccharomyces cerevisiae* Provoked by Cd^{2+}

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The effect of Cd^{2+} poisoning of *Saccharomyces cerevisiae* on ^{45}Ca, ^{109}Cd and [^{14}C]tetraphenylphosphonium (TPP) uptake and cell pH was examined At Cd^{2+} concentrations that produced substantial K^+ efflux the rates of uptake of ^{45}Ca, ^{109}Cd and [^{14}C]TPP increased progressively during incubation of the cells with Cd^{2+}, and the cell pH was lowered concomitantly. The initial rates of uptake of the divalent cations and of TPP were increased in cells pre-loaded with Cd^{2+}, which shows that stimulation of the ion fluxes was exerted by the Cd^{2+} that accumulated in the cells. The distribution of TPP between cells and medium, however, was changed by Cd^{2+}. Although hyperpolarization of the cell membrane by Cd^{2+} cannot be excluded, it is argued that Cd^{2+} primarily stimulated divalent cation uptake by increasing the cation permeability of the cell membrane allowing the cations to enter the cells more easily.

INTRODUCTION

Cd^{2+} is very toxic to all kinds of living cells including yeasts (Norris & Kelly, 1977; Gadd & Mowl, 1983; Kessels *et al.*, 1985; Heldwein *et al.*, 1977; Macara, 1978; Bitton *et al.*, 1984). Not only is growth inhibited but Cd^{2+} also provokes K^+ loss from metabolizing yeast cells (Norris & Kelly, 1977; Gadd & Mowl, 1983, Kessels *et al.*, 1985). Cd^{2+} must first enter the cells before giving rise to a detectable effect (Kessels *et al.*, 1985). Provoking K^+ loss is a property common to a large number of organic poisons, e.g. DIO-9, ethidium, miconazole, trifluoperazine, compound 48/80, calmidazolium and diethylstilboestrol (Foury *et al.*, 1977; Peña & Ramírez, 1976; Peña, 1978, Dufour *et al.*, 1980; Eilam, 1983, 1984; Borst-Pauwels *et al.*, 1983, 1986). These compounds are inhibitors of the yeast plasma-membrane ATPase (Foury *et al.*, 1977; Dufour *et al.*, 1980; Eilam. 1984; Borst-Pauwels *et al.*, 1983, 1986; Serrano, 1980). Furthermore, they all enhance Ca^{2+} or Sr^{2+} uptake. We have now examined whether Cd^{2+} also increases Ca^{2+} uptake.

METHODS

Yeast growth Saccharomyces cerevisiae strain Delft 2 was grown to stationary phase in medium A [1% (w/v) Yeast Extract, 2% (w/v) Bacto-Peptone, 2% (w/v) glucose, 0·2% $MgCl_2$ $6H_2O$, 3·46% (w/v) KH_2PO_4, 0·04% K_2HPO_4, brought to pH 4·5 with HCl] as described by Kessels *et al.* (1985).

Uptake of ^{109}Cd and ^{45}Ca. This was studied by adding 37 nM-^{109}Cd or 1·2 μM-^{45}Ca to a 2% (w/v) yeast suspension in 45 mM-Tris/succinate buffer, pH 5·0, that had been supplemented with 3% (w/v) glucose 20 min earlier The specific activities of ^{109}Cd and ^{45}Ca were 0·9 Ci mmol^{-1} (33·3 GBq mmol^{-1}) and 9·2 mCi mmol^{-1} (340·4 MBq mmol^{-1}), respectively Nitrogen was bubbled through the suspension at 25 °C and at appropriate times 2 ml samples were filtered and washed (Kessels *et al.*, 1985). Radioactivity on the filters was determined by liquid scintillation counting.

Abbreviations. TPP, tetraphenylphosphonium.

Uptake of TPP (tetraphenylphosphonium) This was determined by the procedure of Boxman *et al* (1982) After the 2% (w/v) yeast suspension had been pre-incubated for 20 min with 3% (w/v) glucose in 45 mM-Tris/succinate buffer, pH 5.0, as described above, uptake experiments were started by addition of 0·36 μM-[^{14}C]TPP [specific activity 50 mCi mmol^{-1} (1·85 GBq mmol^{-1})]

Cell pH This was determined by filtering 5 ml samples of 2% (w/v) yeast suspension in Tris/succinate buffer, pH 5·0, washing the cells twice with 2 ml ice-cold water, and freezing the filters in liquid nitrogen (Borst-Pauwels & Dobbelmann, 1972) After thawing, the filters with the cells were boiled for 30 s in 0·5 ml 400 mM-KCl, the pH was then measured with a glass electrode.

Binding of 9-aminoacridine. Binding of this fluorescent dye (1 μM) to 5% (w/v) non-metabolizing yeast cells was measured as described by Theuvenet *et al.* (1984) in 45 mM-Tris/succinate buffer, pH 5·0, at 25 °C in the absence of glucose

Chemicals. 9-Aminoacridine was from Sigma, Bacto-Peptone and Yeast Extract were from Difco, radiochemicals were from Amersham, all other chemicals used were reagent grade and were from commercial sources.

RESULTS

At concentrations of 0·005–1 mM-Cd^{2+} causes K^+ loss from metabolizing yeast cells (Kesseis *et al.*, 1985); we investigated whether this K^+ loss was accompanied by an enhancement of Ca^{2+} uptake. Cd^{2+} (1 mM) gave rise to a much higher uptake of carrier-free ^{45}Ca than was found in the presence of 1 mM non-radioactive Ca^{2+} (Fig. 1). The net rate of ^{45}Ca uptake increased gradually during accumulation of Cd^{2+} reaching a maximum after about 3 min, whereafter it gradually decreased. In the presence of 1 mM non-radioactive Ca^{2+} no increase in the net rate of uptake of ^{45}Ca was observed. Also, the net uptake rate of ^{109}Cd increased gradually during incubation of the cells in the presence of 1 mM non-radioactive Cd^{2+}.

Fig. 1 also shows that the increase in the net uptake rate of ^{45}Ca or ^{109}Cd was due to an increase in the influx rate of the two isotopes rather than to a decrease in the efflux rate. The rates of influx were greatly increased on pre-loading the cells for 5 min with 1 mM-Cd^{2+} before addition of the two isotopes. The net rates of uptake were appreciably lower than the corresponding influx rates. Therefore, there was still appreciable efflux of the two isotopes. The influx rate of ^{45}Ca was not increased on pre-incubating the cells for 5 min with 1 mM-Ca^{2+}.

The dependence of the influx rates of ^{45}Ca or ^{109}Cd on the cellular Cd content showed saturation kinetics (Fig. 2). The maximum increase in the uptake rate was approximately 100%. The cellular Cd content at which half-maximum stimulation occurred was 2·3 mmol (kg dry wt)$^{-1}$.

The compound trifluoperazine not only stimulates K^+ efflux and Ca^{2+} uptake in metabolizing yeast cells but also increases the equilibrium distribution of the lipophilic cation TPP (Eilam, 1983, 1984). This indicates that yeast cells are hyperpolarized by trifluoperazine, which might be the cause of the increased divalent cation uptake provoked by this compound. Similarly, the enhancements of Sr^{2+} and K^+ fluxes by diethylstilboestrol are also accompanied by an increased equilibrium distribution of TPP (Borst-Pauwels *et al.*, 1984) We therefore examined whether Cd^{2+} also enhances TPP uptake. Fig. 3 shows that 1 mM-Cd^{2+} led to a large efflux of TPP from yeast cells which had been pre-loaded to equilibrium with TPP; 1 mM-Ca^{2+} also gave rise to an efflux of TPP. This efflux, however, was much smaller than the efflux provoked by Cd^{2+}.

We considered the possibility that the efflux of TPP was due to a displacement by Cd^{2+} of TPP bound to intracellular binding sites, rather than to a depolarization of the cells. Theoretically, the initial rate of entry of TPP into the yeast cells should not depend on the extent to which TPP is bound inside the cell. Therefore, we also determined the effect of Cd^{2+} on the initial rate of influx of TPP into the cells. The influx rate of TPP uptake into the cells was higher in the presence of 1 mM-Cd^{2+} than in the presence of 1 mM-Ca^{2+} (Fig. 4). Typically, the uptake rate of TPP increased gradually during incubation of the cells with Cd^{2+}. Accordingly, the influx rate of TPP found when TPP was added 5 min after the addition of Cd^{2+} was much greater than the rate found on adding TPP and Cd^{2+} together.

Theoretically, the influx rate depends not only on the membrane potential but also on the surface potential. A reduction in the negative surface potential of the yeast caused by the

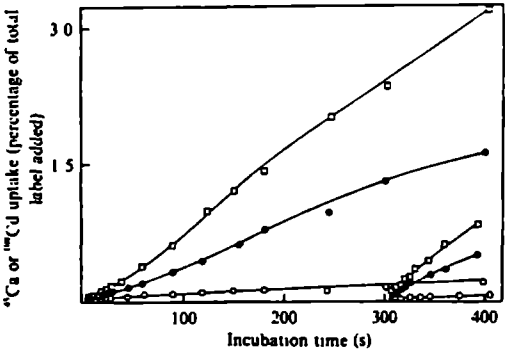

Fig 1. Comparison of ^{45}Ca and ^{109}Cd uptake by metabolizing cells in the presence of 1 mM-Cd^{2+} or 1 mM-Ca^{2+} and effect of pre-loading the cells with Ca^{2+} or Cd^{2+}. □, ^{45}Ca uptake in the presence of 1 mM-Cd^{2+}. ●, ^{109}Cd uptake in the presence of 1 mM-Cd^{2+}; ○, ^{45}Ca uptake in the presence of 1 mM-Ca^{2+}. The carrier-free isotopes were added either together with the non-radioactive divalent cations or 5 min after the addition of these cations.

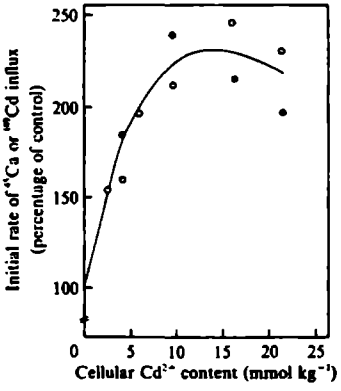

Fig. 2

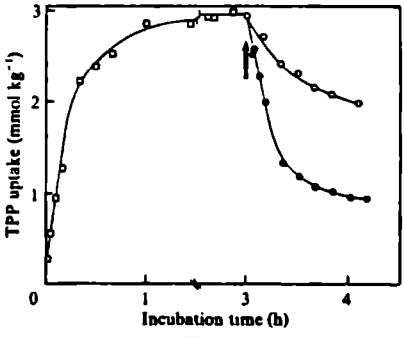

Fig. 3

Fig. 2. Dependence of the influx rate of ^{45}Ca and ^{109}Cd on the intracellular Cd content. The influx rates were obtained by adding the radioactive isotopes after various periods of incubation of the cells with 1 mM-Cd^{2+}. ○, ^{45}Ca uptake, ●, ^{109}Cd uptake.

Fig. 3. Effect of 1 mM-Cd^{2+} or 1 mM-Ca^{2+} on the cellular TPP content of metabolizing cells that had accumulated TPP for 3 h ●, Cd^{2+} added, ○, Ca^{2+} added; □, control (no additions).

addition of divalent cations will lead to a decrease in the influx rate of TPP, because the interfacial concentration of TPP near the plasma-membrane is reduced. Binding of the monovalent cationic dye 9-aminoacridine to the yeast cells is proportional to the interfacial monovalent cation concentration near the plasma-membrane (Theuvenet *et al.*, 1984). The binding of 9-aminoacridine to the yeast cells was reduced more by 1 mM-Cd^{2+} than by 1 mM-Ca^{2+}, namely to 43 ± 5% and 61 ± 5% of the control value, respectively. (Experiments were done in triplicate; values are means ± SEM.) From the 9-aminoacridine binding studies it can be concluded that 1 mM-Cd^{2+} also decreased the interfacial concentration of TPP more than did

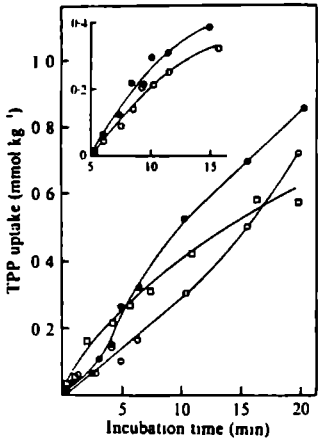

Fig 4

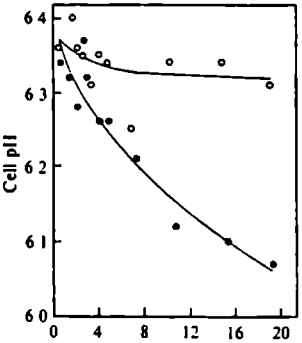

Fig 5

Fig. 4 Uptake of TPP by metabolizing yeast cells – effect of 1 mM-Cd^{2+} or 1 mM-Ca^{2+}. TPP was added either together with divalent cations or after 5 min incubation of the cells with the divalent cations ●, 1 mM-Cd^{2+} added, ○, 1 mM-Ca^{2+} added, □, no divalent cation added. Inset. TPP uptake after 5 min incubation with 1 mM-Cd^{2+}.

Fig 5. Effect of 1 mM-Cd^{2+} (●) or 1 mM-Ca^{2+} (○) on the cell pH of metabolizing yeast.

1 mM-Ca^{2+}, and that the initial rate of TPP influx should be corrected for this differential decrease in interfacial TPP concentration. The corrected influx rates of TPP appeared to be 338 ± 5% (Cd^{2+}) and 161 ± 31% (Ca^{2+}) of the influx rate found in the absence of added divalent cation. This shows that pre-incubation of the cells with Cd^{2+} increased the initial rate of TPP influx more than did pre-incubation with Ca^{2+}. When TPP was added together with the divalent cations, Ca^{2+} did not significantly affect the TPP influx rate, whereas Cd^{2+} increased that rate twofold.

Ethidium, which also causes simultaneous enhancement of K^+ loss and Ca^{2+} uptake (Peña, 1978) leads to an acidification of the cells (Peña & Ramírez, 1976), and 1 mM-Cd^{2+} had a similar effect (Fig. 5). On the other hand, 1 mM-Ca^{2+} did not really lead to a change in cell pH.

DISCUSSION

The property, shared by a large number of organic poisons such as ethidium, trifluoperazine, compound 48/80, calmidazolium, miconazole and diethylstilboestrol (Eilam, 1983; Borst-Pauwels *et al.*, 1986; Serrano, 1980; Borst-Pauwels *et al.*, 1984), of provoking K^+ efflux and concomitantly enhancing Ca^{2+} uptake in metabolizing yeast cells, is also exhibited by Cd^{2+}. This may indicate that Cd^{2+} interferes with yeast cells in a similar way to these organic compounds. All these compounds inhibit the yeast plasma-membrane ATPase (Dufour *et al.*, 1980, Eilam, 1984; Borst-Pauwels *et al.*, 1986; Serrano, 1980), as does Cd^{2+} (Ahlers & Rösick, 1985)

Whether the hyperpolarization of the plasma-membrane is a common effect of the organic poisons is less certain. Until now, only trifluoperazine and diethylstilboestrol have been proven to cause hyperpolarization (Eilam, 1984; Borst-Pauwels *et al.*, 1984, Eilam *et al.*, 1985). The other organic poisons, DIO-9, ethidium, miconazole, compound 48/80 and calmidazolium, do not increase the intracellular concentration of TPP at equilibrium; they only increase the influx rate of TPP (G. W. F. H. Borst-Pauwels, unpublished). The efflux of TPP from cells pre-loaded with TPP, as provoked by Cd^{2+}, may be due to a displacement by Cd^{2+} of TPP bound to

intracellular binding sites, rather than to depolarization of the cells. Some support for this view is provided by the fact that the initial rate of influx of TPP into the cells in the presence of 1 mM-Cd^{2+} is higher than that in the presence of 1 mM-Ca^{2+}.

A hyperpolarization of the plasma-membrane may account for the increase in the rate of ^{45}Ca uptake caused by Cd^{2+}. On the other hand, the increase in TPP uptake observed after 5 min pre-incubation of the cells with 1 mM-Ca^{2+} is not accompanied by an increased rate of ^{45}Ca influx. This shows that an increase in TPP influx rate is not necessarily accompanied by an increase in the rate of divalent cation uptake. Furthermore, as will be shown elsewhere, the increase in Ca^{2+} uptake provoked by trifluoperazine, a compound which hyperpolarizes the yeast cells (Eilam, 1984), is not due to this hyperpolarization but to an increase in the cation permeability of the cells. Therefore, an alternative explanation for the increased rate of ^{45}Ca influx provoked by Cd^{2+} is that the permeability of the cells is increased by Cd^{2+}. That permeabilizing the cells can lead to increased cation uptake is shown by the fact that DEAE-dextran, which permeabilizes the plasmalemma specifically without affecting the tonoplast, also increases the influx rate of Ca^{2+} (Theuvenet *et al.*, 1986). A third possible explanation for the enhancement of ^{45}Ca uptake by 1 mM-Cd^{2+} is the acidification of the cells caused by Cd^{2+}. A decrease in cell pH does indeed lead to an increase in divalent cation uptake (Roomans *et al.*, 1979).

As we have shown (Kessels *et al.*, 1985) the dependence of the K^+ efflux provoked by Cd^{2+} on the intracellular Cd^{2+} content exhibits saturation kinetics. We have now shown that the dependence of the rate of ^{109}Cd or ^{45}Ca influx on the intracellular Cd content also shows saturation kinetics. This supports the notion that saturable sites are involved in the interaction of Cd^{2+} with the yeast cell. Neither the precise location inside the cells nor the nature of the binding sites are known at this stage. The similarity in the dependence of K^+ efflux on the cellular Cd content and the dependence of Ca^{2+} influx on cellular Cd supports the view that both processes are closely related, in accordance with earlier findings (Kessels *et al.*, 1985). The half-maximum Cd^{2+} concentrations are approximately the same.

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CHAPTER 4

DO Cd BINDING PROTEINS PLAY A ROLE IN THE DEVELOPMENT OF RESISTANCE AGAINST Cd IN YEAST ?

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(submitted to The journal of General Microbiology)

SUMMARY

In both Schizosaccharomyces pombe L 972(h⁻) and Saccharomyces cerevisiae Delft 2 resistance against 2 mM $CdCl_2$ could be developed by growing the cells at step-wise increased concentrations of $CdCl_2$ with each medium transfer. Cd resistant cells of S. pombe which grew in the absence of Cd^{2+} did not possess Cd binding proteins, whereas these cells were still insensitive to high $CdCl_2$ concentrations. Addition of 1 mM $CdCl_2$ to these cells in the late exponential growth phase led to the synthesis of Cd binding proteins. This was also true for wild type cells. Still these cells could not grow in the presence of $CdCl_2$. Apparently Cd binding proteins did not play a crucial role in the development of Cd resistance in S. pombe. Neither wild type cells nor Cd resistant cells of S. cerevisiae could synthesize Cd binding proteins. Addition of 1 mM $CdCl_2$ to late exponential phase cells of both S. cerevisiae strains did not lead to the synthesis of Cd binding proteins. Furthermore inoculating the Cd resistant cells in 2 mM $CdCl_2$ containing medium did not lead to synthesis of Cd binding proteins, though the cells grew very well. Therefore it may be concluded that in S. cerevisiae resistance against Cd did not depend upon synthesis of Cd binding proteins.

INTRODUCTION

In the yeast Saccharomyces cerevisiae a copper binding metallothionein has been detected during Cu poisoning and the corresponding gene has been cloned (Fogel et al., 1983, Butt et al., 1984). In yeast copperthionein has a similar function as in monkeys. Yeast cells, lacking metallothionein-like copper binding protein and the corresponding gene, could regain copper resistance by means of complementation with monkey DNA, coding for copperthionein (Thiele et al., 1986).

Whether in yeast resistance against Cd^{2+} also is related to synthesis of cadmium binding proteins, is less clear. In Schizosaccharomyces pombe L 972(h⁻), synthesis of Cd binding proteins, Cd BP I and Cd BP II, could be induced by the presence of 1 mM $CdCl_2$ in the medium (Murasugi et al., 1981, Murasugi et

al., 1984, Kondo et al., 1984 and Hayashi et al., 1986). Recently Grill et al., 1986 reported about five additional Cd binding proteins that were synthesized in cells of S. pombe exposed to Cd^{2+}. The, now in total seven, Cd BP's, carrying the new name of phytochelatins, are commonly found in higher plants (Grill et al., 1986). Phytochelatins are small sulfur-rich homologous peptides that bind heavy metals. The nature of these phytochelatins greatly differs from the Cd^{2+} complexing "genuine" metallothioneins (Vallee, 1979) found in animals.

Hayashi et al., 1986 and Joho et al., 1986 developed Cd resistant strains of both S. pombe L 972(h^-) and S. cerevisiae 101N. They suggest that the resistance against Cd was due to two factors, a decrease in Cd^{2+} uptake and formation of Cd binding proteins by these cells. In this study we examined the possible role and appearance of Cd binding proteins in Cd resistant strains of both S. cerevisiae Delft 2 and S. pombe L972(h^-).

METHODS

Organisms and culture conditions. The diploid yeast strains S. cerevisiae Delft 2 and S. pombe L 972(h^-) were grown in YEP medium (1% w/v Yeast Extract, 2% w/v glucose and 2% w/v Bacto-Peptone) on an orbital shaker at 125 rpm in 250 ml batch cultures at 30 °C. Cd resistant strains of both yeasts were obtained as follows. We started growing wild type cells in medium containing 1 µM $CdCl_2$ and doubled the $CdCl_2$ concentration with each new inocculation untill a final concentration of 2 mM was reached. Above that concentration precipitation of Cd^{2+}-complexes with medium constituents occurred. After inoculation of 250 ml Cd^{2+} free YEP medium with 0.5 ml cell suspension obtained from a stationary culture, growth reached the late exponential phase 20 h later. This procedure was repeated twice. Then cultures of the Cd resistant strains of S. cerevisiae and S. pombe were considered to be virtually free of $CdCl_2$. The Cd resistant cells of both strains appeared to be still resistant against Cd^{2+} when eight transfers to Cd free medium had occurred.

Induction of Cd binding proteins. For studying the effect of Cd^{2+} upon the synthesis of Cd binding proteins, two procedures

were used. 1) Cd binding proteins were induced by addition of 0.25 ml from a sterile 1 M $CdCl_2$ stock solution to 250 ml suspension of the late exponential cells in YEP medium. Cells were harvested for protein extraction 20 h later. In control experiments an equivalent amount of sterile water was added to the YEP medium instead of the $CdCl_2$ solution. 2) 0.25 ml suspension of Cd resistant cells of S. pombe and S. cerevisiae were inoculated in YEP medium with 2 mM $CdCl_2$ present already. After 20 h of growth, the cells were harvested for protein extraction.

Extraction and chromatography of Cd binding proteins. After the 20 h of incubation with $CdCl_2$, the cells were harvested by centrifugation, washed twice with 250 ml distilled water and resuspended in 5 ml of elution buffer (50 mM Tris/HCl, 100 mM KCl pH 7.6). Pestle, mortar and glass pearls (0.45 mm Ø) were used to homogenize the cells at 0 °C. The homogenate was centrifuged at 49,000 x g for 20 min at 4 °C in a SS 34 Sorvall rotor. The resulting water-soluble extract was immediately applied to a Sephadex G 50 fine column (Ø 1.6 cm, height 29 cm, void volume 21.9 ml as determined with Dextran Blue of 2×10^6 D.). Elution of the proteins proceeded with 7.5 ml elution buffer per hour. 3 ml fractions were collected under continuous measurement of light absorption at 254 nm. The fractions were examined with an atomabsorption spectrophotometer (AAS; Beckman 1272) for their Cd^{2+} contents.

Labeling of binding proteins with ^{35}S cystein. 100 µCi ^{35}S cystein was added to 250 ml nutrient medium during the incubation of the late exponential cells in the presence of 1 mM $CdCl_2$. Extraction and chromatography of Cd binding proteins were carried out in a similar way as described above. The gel filtration fractions were examined for radioactivity by means of liquid scintillation.

All experiments were carried out at least in duplicate.

Chemicals. Molecular weight calibration of the Sephadex G 50 fine column was carried out with Protein mixture nr 5 (6.500-29.000 Dalton) from Serva. ^{35}S-cystein (MW 157.7, specific activity 53.6 mCi/nmol) was purchased from Amersham. Yeast Extract and Bacto Peptone were from Difco. All other chemicals were reagent grade and obtained from commercial sources.

Cd binding proteins in S. pombe L 972(h⁻)

In agreement with the results of Hayashi et al., 1986, we were able to induce Cd binding proteins in the wild type strain of S. pombe L 972(h⁻). Fig. 1a and 1b show elution patterns obtained with cell extracts from S. pombe wild type cells. For Fig. 1a the wild type cells were induced to synthesize Cd binding proteins by exposing the late exponential cell culture to 1 mM $CdCl_2$ for 20 h. The results of the control experiment (an equivalent amount of water was added to the late exponential culture instead of a $CdCl_2$ solution and an appropriate amount of $CdCl_2$ was added to the cell extract before chromatochraphy) are shown in Fig. 1b. The peaks around an elution volume of 47 ml in both 254 nm absorption and Cd content of the eluted fractions in Fig. 1a, were caused by Cd binding proteins present in the fractions concerned.

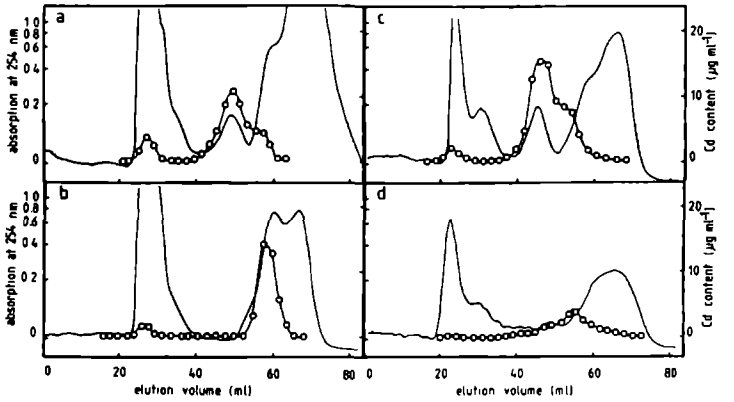

FIG 1: Sephadex G 50 fine chromatography of water-soluble proteins from S. pombe wild type (1a,1b) and its Cd resistant strain (1c,1d). After growth in Cd-free YEP medium for 20 h, the cells were exposed to 1 mM $CdCl_2$ during an additional 20 h in the same medium (1a,1c). In the control experiment the cells were not exposed to $CdCl_2$ during the additional incubation (1b,1d). In the latter case an appropriate amount of $CdCl_2$ was added to the cell extracts before chromatography. In all cases extinction at 254 nm was registrated continuously (full drawn line) and the Cd content of the eluted fractions (o) was determined.

When growing the S. pombe wild type cells in the absence of 1 mM $CdCl_2$, Cd binding proteins were not present, see Fig. 1b. Furthermore Fig. 1a and 1b show that the fractions between 22 and 36 ml elution volume contained proteins of high molecular weight with some Cd^{2+} bound to them. Cd binding was higher when the cells were grown in the presence of Cd^{2+} than in the absence of Cd^{2+}. The fractions between 56 and 78 ml elution volume contained proteins of low molecular weight. Hardly any Cd^{2+} was detected in these fractions. Free Cd^{2+} was eluted at a volume of about 58 ml as determined by fractionation of a $CdCl_2$ solution alone (data not shown). This indicates that, in the Cd content pattern of Fig. 1a, the shoulder on the right side of the Cd binding protein peak was caused by free Cd^{2+}. The same argument holds for the peak in Cd content around an elution volume of 58 ml in Fig. 1b.

Fig. 1c shows that the Cd resistant strain of S. pombe 972 (h^-) also was able to synthesize Cd binding proteins. When late exponential cells were exposed to 1 mM $CdCl_2$ for 20 h, gel filtration of their protein extract showed the characteristic peaks of Cd binding proteins in both the 254 nm absorption and the Cd content pattern of the eluted fractions. The general patterns of the 254 nm absorption and the Cd content were similar to those found for wild type cells, see Fig. 1a. There was also some Cd^{2+} bound to heigh molecular weight compounds. In the Cd resistant cells the amount of Cd binding proteins being synthesized was somewhat higher than in the wild type cells. That Cd resistant cells of S. pombe only synthesized Cd binding proteins when induced with $CdCl_2$, is shown in Fig. 1d. No peaks of Cd binding proteins in the 254 nm absorption and the Cd content patterns could be detected when the Cd resistant cells of S. pombe had not been exposed to $CdCl_2$.

It is well known that Cd binding proteins have a high cystein content. Murasugi et al., 1981 showed that ^3H cystein was incorporated into the Cd^{2+} induced Cd binding proteins of S. pombe. We have confirmed this for S. pombe wild type cells using ^{35}S cystein (data not shown). Furthermore we examined whether Cd resistant cells of S. pombe also showed cystein incorporation in a protein fraction, induced by the addition of Cd^{2+}. This appeared to be true, see Fig. 2a. The peak of ^{35}S cystein radioactivity coincided with the peak in the 254 nm absorption

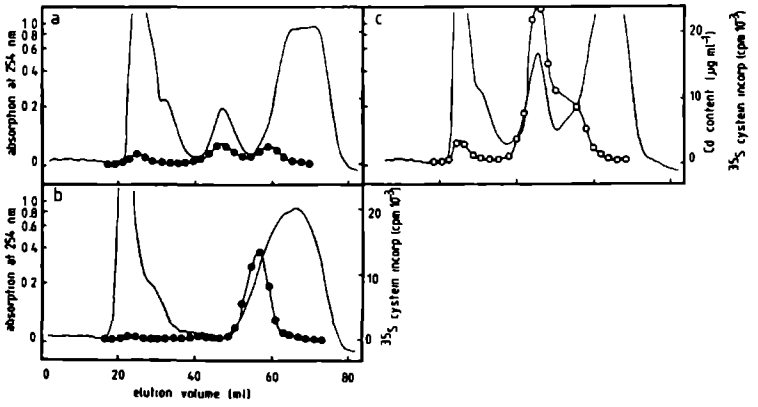

FIG 2: Sephadex G 50 fine chromatography of water-soluble proteins from Cd resistant S. pombe cells, grown in YEP medium. Late exponential cells were exposed to 1 mM $CdCl_2$ (2a). In the control experiment, 2b, the cells were not exposed to $CdCl_2$ and an appropriate amount of $CdCl_2$ was added to the protein extract. Under both circumstances ^{35}S cystein was present and it's incorporation into the fractionated proteins (●) was determined. Cells of 1c were inoculated in 250 ml YEP medium containing 2 mM $CdCl_2$ and allowed to grow for 20 h before extraction was carried out. See further subscription to Fig. 1.

around an elution volume 47 ml. In addition we showed that the Cd^{2+} induced ^{35}S cystein peak was absent in extracts of Cd resistant S. pombe cells that had not been exposed to 1 mM $CdCl_2$, see Fig. 2b. This confirms that Cd binding proteins were only synthesized when the cells had been exposed to 1 mM $CdCl_2$ for 20 h. Fig. 2a and 2b also show that some ^{35}S cystein was incorporated in the high molecular weight protein fractions around an elution volume of 29 ml. This radioactivity clearly was higher in the Cd^{2+} exposed cells than in the control cells not being exposed to 1 mM $CdCl_2$. The radioactivity around an elution volume of 67 ml on the other hand was much higher for the control cells. Apparently the content of free cystein was higher with the control cells.

The late exponential cells hardly grew during the 20 h incubation. Wild type cells did not grow at all in the presence of

$CdCl_2$ and the number of Cd resistant cells per ml medium at maximum doubled in the presence of $CdCl_2$. The latter was also true for the cells incubated in the absence of added $CdCl_2$. We now examined wether Cd binding proteins were also synthesized by exponentially growing cells. A small amount of Cd resistant cells of S. pombe was inoculated in YEP medium containing 2 mM $CdCl_2$. After 20 h of growth, when the culture had reached the late exponential phase, the cells were harvested and analyzed for the presence of binding proteins. Fig. 2c shows that under these conditions synthesis of Cd binding proteins also occurred during the 20 h of exponential growth. The patterns in 254 nm absorption and Cd content again showed the characteristic peaks of Cd binding proteins around an elution volume of 47 ml.

The elution volumes of five marker proteins were used to determine the molecular weight of the Cd binding proteins synthesized in S. pombe wild type and Cd resistant cells. The molecular weight appeared to be approximately 5600 Dalton.

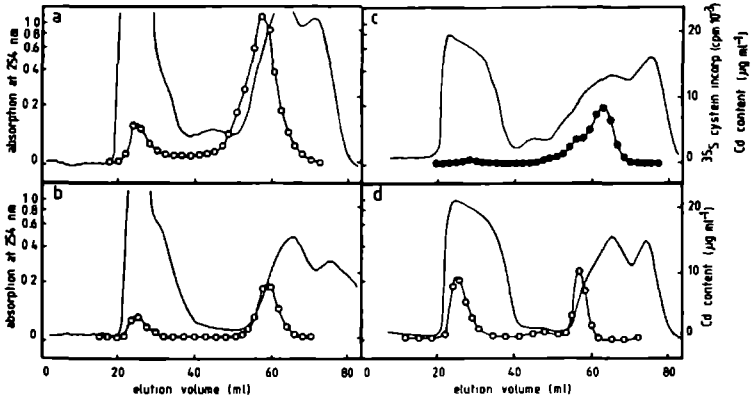

FIG 3: Sephadex G 50 fine chromatography of water-soluble proteins from the wild type (3a) and the Cd resistant strain (3b,3c and 3d) of S. cerevisiae Delft 2. Late exponential cells grown for 20 h in YEP medium in the absence of $CdCl_2$, were exposed to 1 mM $CdCl_2$ in the YEP medium for another 20 h. In the case of experiment 3c ^{35}S cystein (●) was present. For 3d the cells were inoculated in YEP medium with already 2 mM $CdCl_2$ present and were grown for 20 h. See further subscription to Fig. 1.

Fig. 3a shows the results of G 50 fine Sephadex gel filtration with a protein extract from S. cerevisiae wild type cells. After growing untill the late exponential phase, the cells were exposed to 1 mM $CdCl_2$ for 20 h. Neither the 254 nm absorption pattern nor the pattern of Cd content in the eluted fractions showed peaks different from those present in patterns obtained with wild type cells that had not been exposed to $CdCl_2$. Just as was found with S. pombe, some Cd^{2+} was bound to heigh molecular weight proteins around an elution volume of 29 ml. These Cd binding compounds, however, were also found with extracts of wild type cells of Delft 2 that had not been exposed to $CdCl_2$ (data not shown).

Also in the extracts obtained after exposing late exponential cultures of the Cd resistant S. cerevisiae to 1 mM $CdCl_2$ for 20 h, we were not able to detect Cd binding proteins, (Fig. 3b and 3c). This was confirmed in parallel experiments carried out with late exponential cells which were exposed to 1mM $CdCl_2$ and to 100 uCi ^{35}S cystein for 20 h. No peak in the radioactivity appeared around the position at which Cd binding proteins were expected to show up, see Fig. 3c. Finally, growth of Cd resistant Delft 2 cells in medium that already contained 2 mM $CdCl_2$ at the moment of inoculation, neither resulted in synthesis of Cd binding proteins , as is shown in Fig. 3d.

DISCUSSION

Although wild type cells of S. pombe can be induced to synthesize Cd binding proteins by exposure to 1 mM $CdCl_2$ in the medium, their growth nevertheless is stopped completely. As a matter of fact growth of S. pombe is already affected at Cd concentrations as low as 1 uM (data not shown). Apparently synthesis of Cd binding proteins does not give rise to resistance against Cd in wild type cells of S. pombe. It is not likely that the extremely high degree of Cd resistance in the Cd resistant cells of S. pombe, is due to the fact that they are able to synthesize a somewhat larger amount of Cd binding proteins than the wild type cells. Moreover, the lower amount of Cd binding proteins synthesized in

wild type cells may be apparent because part of the cells may be already dead, due to exposure of the cells to Cd^{2+}.

For a Cd accomodated strain of S. cerevisiae 101 N, Joho et al.,1985a, have shown that the decrease in Cd sensitivity is accompanied by the formation of Cd binding proteins. This is also true for S. cerevisiae strain 301 N, a Cd resistant mutant of strain 101 N. Possibly in these strains of S. cerevisiae synthesis of Cd binding proteins does contribute to the decrease in Cd sensitivity. Furthermore uptake of Cd^{2+} is decreased (Joho et al., 1985b), which also may contribute to the resistance against Cd^{2+}.

Comparing the S. cerevisiae wild type strain with the Cd resistant strain, we also observe a small reduction in Cd^{2+} uptake (data not shown). But no Cd binding proteins could be demonstrated in our wild type strain of S. cerevisiae on adding 1 mM $CdCl_2$ to late exponential cells. Grill et al., 1986, Macara, 1978 and Murasugi et al., 1981, neither could demonstrate Cd binding proteins in S. cerevisiae. It appeared that also with the Cd resistant strain of Delft 2 synthesis of Cd binding proteins could not be detected.

The experimental approach applied by Joho, 1985a, 1985b and 1986, used chitosan in order to permeabilize the cells and a synthetic medium for growing the cells. We checked whether the differences in resuls, found by Joho and by us, may be due to the differences in the experimental procedures. However, on applying exactly the growth and extraction procedures of Joho et al., 1986, still Cd binding proteins in both the wild type and the Cd resistant strain of S. cerevisiae were not found.

Our results obtained with wild type and Cd resistant cells of both S. cerevisiae and S. pombe, indicate that synthesis of Cd binding proteins is not essential for the development of resistance against high Cd^{2+} concentrations in the growth medium. The Cd resistant strain of S. cerevisiae Delft 2 can grow very well in nutrient medium supplied with 2 mM $CdCl_2$ without synthesis of binding proteins. On the other hand, the wild type strain of S. pombe can synthesize Cd binding proteins as pointed out above but this strain still is extremely sensitive to $CdCl_2$. Furthermore the Cd resistant strain of S. pombe L 972(h^-) apparently does not posses an increased capacity for synthesis of Cd binding

proteins. Thus there are no indications that Cd binding proteins play a crucial role in the development of resistance against Cd.

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CHAPTER 5

A ^{31}P-NMR STUDY AND CHEMICAL ANALYSIS OF PHOSPHATE DISTRIBUTION IN Cd SENSITIVE AND Cd RESISTANT YEAST STRAINS

SUMMARY

The distribution and composition of phosphorus compounds from wild type and Cd resistant strains of Saccharomyces cerevisiae Delft 2 and Schizosaccharomyces pombe L 972(h⁻) were studied by means of ^{31}P-NMR resonance and by chemical analysis. The polyphosphate content of the Cd resistant strains appeared to be significantly smaller than that of the wild type cells. The differences in polyphosphate content of the cells were most pronounced in the S. cerevisiae strain where the polyphosphate content of the wild type cells was at least twice that of the Cd resistant cells. Cd resistant cells of S. pombe contained 20 to 30% less polyphosphate than the corresponding wild type cells. The stronger agglutination of the Cd resistant cells of both yeast strains was probably not due to an increased phosphomannan content of their cell walls.

INTRODUCTION

Besides the various nucleoside phosphates, the condensed inorganic polyphosphates, found in many cell-types, also play an important role in cellular bioenergetics (Kualev, 1983). Inorganic polyphosphates (poly P) are linear polymers in which orthophosphate residues are linked by energy-rich phospho-anhydride bonds (Kulaev and Vagabov, 1983). The number of phosphate residues in inorganic poly P in vivo, may vary from two in pyrophosphate to several thousands in high molecular weight polyphosphates (Kulaev, 1979).

Poly P is widely distributed among microorganisms. In yeast the phase of growth and the phosphate content of the medium are important for biosynthesis of poly P. During the exponential phase of growth, the poly P content in yeast cells is relatively low. When growth slows down, the poly P content increases gradually. In yeast poly P may accumulate to even 20% of the cell dry weight. Such a "polyphosphate overplus" can occur in non-growing cells (Kulaev and Vagabov, 1983).

Poly P can occur in various compartments of the yeast cell. Jaspers and van Steveninck, 1975 and Tijssen et al., 1980, reported about highly polymerized polyphosphates localized in the periplasmic

compartment between plasmamembrane and cell wall. During logarithmic growth this highly polymerized surface poly P fraction even accounted for about 40% of the total amount of poly P present in the cells. In stationary phase cells, however, the highly polymerized surface fraction accounted for only 9% of the total poly P (Tijssen et al., 1980, Tijssen and van Steveninck, 1984). In 1968 Indge was the first to report about the occurrence of poly P in yeast vacuoles. Matile, 1978 and Wiemken et al., 1979 were able to isolate intact vacuoles from yeast protoplasts and thereby they could study poly P distribution in yeast cells.

The physiological role of poly P in yeast cells still is a matter of discussion. Probably they are involved in the regulation of intracellular metabolites like nucleoside phosphates, and orthophosphate (Pi). Furthermore formation of poly P may serve as a source of stored energy and orthophosphate. In this study we investigated wether in yeast, resistance against Cd may be accompanied by a change in phosphate composition or distribution in the cells. The underlying thought was that especially poly P might function as high molecular weight ion exchanger (Kulaev and Vagabov, 1983) and thereby contribute to the decrease of free cellular Cd^{2+}, that is toxic to the cells. For that purpose we compared phosphate distribution in wild type and Cd resistant strains of both Saccharomyces cerevisiae Delft 2 and Schizosaccharomyces pombe L 972 (h^-).

Another feature we examined, was whether there was a relationship between Cd resistance and phosphomannan content of the cell wall. The Cd resistant strains of S. cerevisiae and S. pombe do display a high degree of agglutination and can be classified as flocculent yeast strains, whereas the wild type strains are non-flocculent. Mill, 1966, reported about an increased P-mannan content in cell walls of flocculent yeast strains. We have now studied whether the phosphomannan content in Cd resistant cells also is increased.

MATERIALS AND METHODS

Organisms and culture conditions. The diploid yeast strains Saccharomyces cerevisiae Delft 2 and Schizosacharomyces pombe L

972(h^-) were grown in 250 ml batch cultures of medium A on an orbital shaker at 125 r.p.m. and at 30°C. Medium A was composed of 1% w/v Yeast Extract, 2% w/v Bacto Peptone, 2% w/v glucose, 0.2% w/v $MgCl_2.6H_2O$, 3.46% w/v KH_2PO_4, and 0.4% w/v K_2HPO_4, brought to pH 4.5 with HCl.

The Cd resistant strains of both yeasts were obtained by inoculation of wild type cells in medium starting with a Cd content of 1 µM. With each transfer into fresh medium, the concentration of $CdCl_2$ was doubled untill a final content of 2 mM $CdCl_2$ was reached.

For ^{31}P-NMR experiments, stationary cells were harvested after 48 h of growth by centrifugation. They were washed twice with distilled water and resuspended in 45 mM Tris/succinate provided with 10% v/v D_2O. at a cell density of 40% wet weight per volume. Chemical phosphate analysis also was carried out with stationary cells, grown for 48 h and harvested from the nutrient medium by centrifugation followed by two washing steps with distilled water. The cell pellets were used for chemical analysis of the phosphate composition

^{31}P-NMR. A Bruker WM200 spectrometer was used operating in the Fourier transform mode at 81.01 MHz with broad band ^{1}H decoupling at 200 MHz. The pulse length was 12 µs with a relaxation delay of 1 s and a sweep width of approximately 8000 Hz. Chemical shifts are given relative to trimethylphosphate (TMP) used as external reference. In some cases methylphosphonate (MEP) was used as internal standard. All reported shifts are denoted according to the δ-convention, i.e. positive values represent shifts to lower field. The samples containing 10% D_2O had a volume of 15 ml and were measured in Wilmad tubes with a diameter of 20 mm. The spectra represent a time average of 50 or 100 scans and were recorded at 22 °C.

Chemical analysis of phosphate composition. The procedure for chemical analysis of the phosphate composition used in this work, partly was adapted from Katchman and Fetty (1955) and partly from Ehrenberg (1961). After centrifugation, cell pellets of about 600 mg dry weight were extracted with 10 ml 15% trichloric acid (TCA) at 4 °C for 1 h. After centrifugation the residue was extracted twice with 10% TCA for 1 h. The three TCA extracts were combined and denoted fraction 1. The residue obtained after TCA extraction was

extracted twice with 10 ml of 97% ethanol for 30 min at 30° C and after that three times with 5 ml ethanol-ether (3:1) mixture for 3 min at 30° C. These five extracts were combined. The ethanol and ether were evaporated by mild heating and passing through nitrogen. The residue was resuspended in concentrated H_2SO_4 and denoted fraction 2. The next step consisted of an extraction of the residue with 10 ml 1N NaOH at 30° C followed by two washings with 1 ml 1N NaOH each. The combined extracts were indicated fraction 3. Finally the residue was extracted with 5 ml 1 N HCl for 5 min at 100° C and with 1 ml 1 N HCl for 5 min at 100° C. The combined HCl fractions were indicated as fraction 4.

During the whole procedure, all extracts were kept at 0° C when possible. After combination of extracts, the fractions were immediately neutralized and stored at -20° C. Pi was determined in all four fractions according to Fiske and Subbarow (1926). Besides that, a part of fraction 1 was brought to 1 N HCl, and heated to 100° C. After 7 min and 30 min at 100° C samples were withdrawn and neutralized for Pi determination. The increase in Pi content after 7 min 100° C was mainly caused by degradation of labile low molecular weight TCA-extractable poly P. The increase in Pi content after 30 min at 100° C represented high molecular weight poly P. All Pi measurements were calculated back to the dry weight of yeast cells from which the extracts originated.

Yeast Extract and Bacto Peptone were purchased from Difco, All other chemicals were reagent grade and obtained from commercial sources.

RESULTS

The ^{31}P-NMR spectrum of S. cerevisiae wild type cells, 10 min after glucose administration, is presented in Fig. 1. The relevant resonances were denoted in this figure and it clearly showed the very pronounced peak of the poly P resonance at -26.3 ppm with resonances of the corresponding ultimate and penultimate phosphate groups at -10.1 and -24.5 ppm, respectively. Furthermore peaks of phosphomannan at -4 7 ppm, vacuolar Pi at -2.7 ppm, cytosolic Pi at -1 5 ppm, and the external reference TMP were

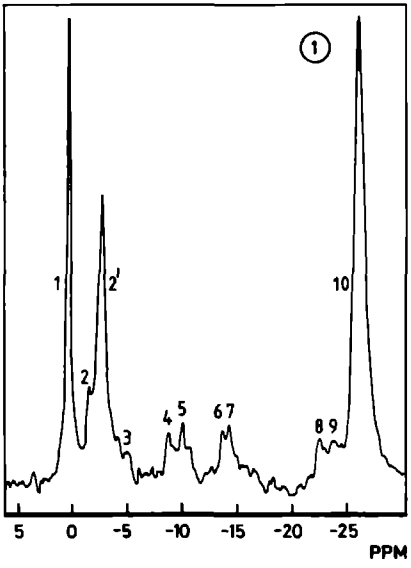
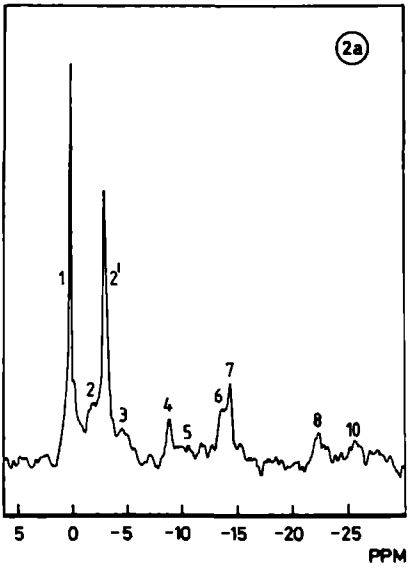
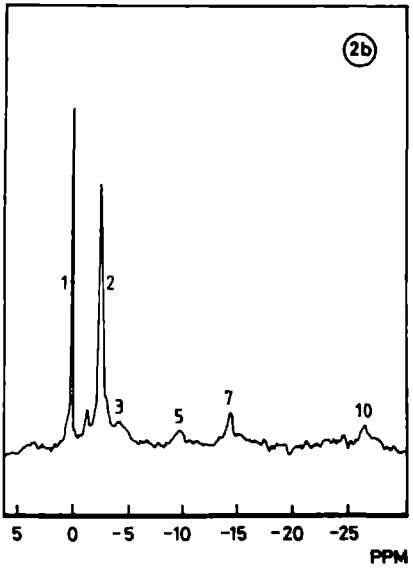


FIG 1. ^{31}P-NMR spectrum of S. cerevisiae Delft 2 wild type cells 10 min after addition of glucose (5% w/v) to a 40% w/v suspension of intact cells in Tris-succiate buffer pH 5.0. 50 scans were accumulated. Peak assignments: peak 1, external TMP reference; peak 2, cytoplasmatic P_i; peak 2', vacuolar P_i; peak 3, P-mannan; peak 4, γ-ATP; peak 5, terminal P of poly P_i; peak 6, α-ATP; peak 7, NAD; peak8, β-ATP; peak 9, penultimate P of poly P_i; peak 10, poly P.





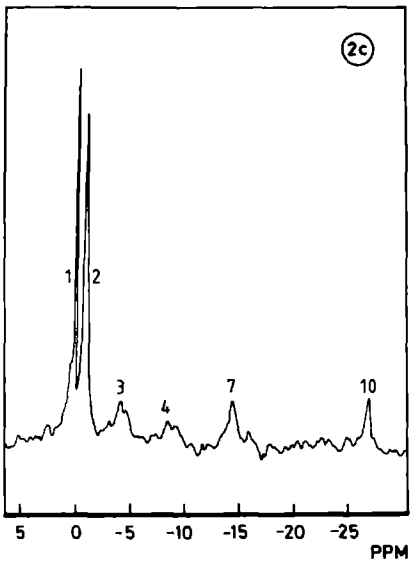


FIG 2. ^{31}P-NMR spectra of Cd resistant S. cerevisiae Delft 2 cells 15 min after addition of glucose (5% w/v). Fig 2a: 50 scans of the suspension of intact cells (40% w/v) in Tris-succinate buffer pH 5.0 were accumulated for this spectrum. Fig 2b, the same cell suspension after boiling for 5 min in medium pH 6.15. Peak 2 is *orthophosphate*. Fig 2c, the medium pH of the boiled suspension was raised to 7.5. See further subscript to Fig. 1.

present.

In Cd resistant cells the poly P content was much lower. Fig. 2a shows the ^{31}P-NMR spectrum of Cd resistant S. cerevisiae cells with a low poly P content. From the Pi peak at -2.8 ppm the existence of vacuolar compartments in the Cd resistant cells could be concluded. At a resonance of -4.7 ppm the P-mannan peak was present and apparently it's surface was of the same order of magnitude as that of the P-mannan peak in the spectrum of the wild type cells. We have now examined wether the lower poly P content was due to complexation of the poly P to cell constituents. For that purpose the cells were boiled and subsequently the pH of the medium was raised. Fig. 2b shows the spectrum after boiling the cells and setting the medium pH to 6.15. A very small peak became visible at -26.3 ppm indicating the presence of polyphosphate. When the pH of the boiled suspension of the Cd resistant S. cerevisiae cells was brought to 7.5, the poly P peak at -26.3 ppm became more pronounced, see Fig. 2c. The Pi peak in the spectrum of the boiled Cd resistant cells shifted towards the direction of 0 ppm. There was no change in the position of the P-mannan peak.

Table 1 shows the results of a quantitative analysis of

^{31}P NMR spectroscopy with wild type and Cd resistant cells of S. cerevisiae strain Delft 2, using 5 mM MEP as internal standard. In the cell cultures used in this experiment, the poly P content of the wild type cells was more than eight times higher than that of the Cd resistant cells. Both the content of sugar phosphates and the content of Pi, as calculated from the peak intensities in the NMR spectra, were higher in the wild type cells. The ratio was significantly higher than 1. For NAD^+ (NADH, $NADP^+$ and NADPH) the reverse was found.

	peak intensity (mmol kg dry wt^{-1})		ratio $\frac{W.T.}{Cd\ res.}$
	wild type	Cd resistant	NMR data
cytosolic + vacuolar Pi	132 ± 24	90 ± 24	1.58 ± 0.21
poly P	261 ± 68	48 ± 29	8.41 ± 2.86
NAD	24 ± 7	33 ± 10	0.73 ± 0.02
sugar P	37 ± 10	32 ± 9	1.19 ± 0.05

Table 1. Quantitative results of ^{31}P NMR spectroscopy with wild type and Cd resistant cells of S. cerevisiae in the absence of glucose. Peak areas were used to calculate phosphate contents in mol per kg dry weight. The internal MEP standard served as the reference. The density of the yeast suspension was 40% w/v. The experiment was carried out in triplicate. Standard errors of the mean are given. The ratios were calculated for each separate experiment and were then averaged.

Fig. 3 shows ^{31}P-NMR spectra of wild type (3a) and Cd resistant (3b) cells of S. pombe. The poly P content of the Cd resistant cells appeared to be smaller than that of the wild type cells, though less pronounced as in S. cerevisiae. The reduction amounted to 35%.

The results of the chemical analysis, carried out with wild type and Cd resistant cells of both S. cerevisiae and S. pombe, are shown in table 2. Fraction 1 consisted of orthophosphate (1a), labile low polymer poly P (1b) and relatively more stabile, though still acid

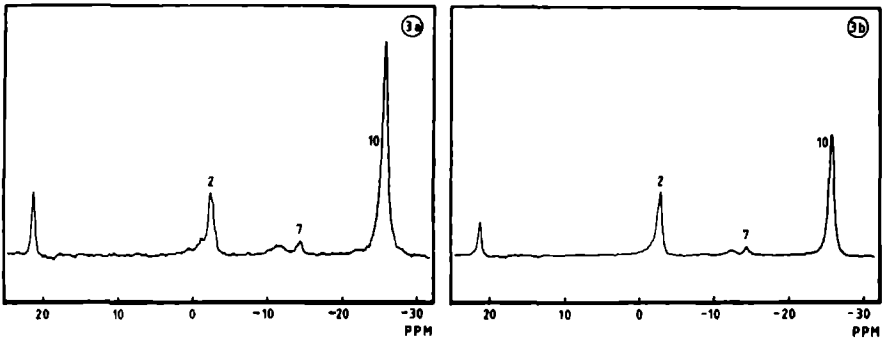


FIG 3. ^{31}P-NMR spectra of 40% w/v suspensions of S. pombe wild type cells (Fig 3a) and Cd resistant cells (Fig 3b) without addition of glucose. 5 mM MEP was used as an internal standard (peak 11) See further subscript to Fig. 1.

	TCA soluble phosphate			TCA insoluble phosphate		
	ortho P	labile low M W poly P	stable high M W poly P	lipid P	TCA insoluble poly P	rest P DNA, RNA, sugar P, P mannan
S cerev wild type	108 4±38	1170±44	111 6±27	82±11	31±01	98 4±110
S cerev Cd resist	805±73	652±94	593±115	82±10	28±06	227±10
S pombe wild type	974±83	963±64	809±69	70±03	23±02	118±09
S pombe Cd resist	836±47	664±60	603±96	93±04	16±01	109±30

Table 2. Results of the chemical analysis of S. cerevisiae Delft 2 and S. pombe L 972(h-) wild type and Cd resistant cells are shown. The cells were harvested from the nutrient medium by centrifugation, washed twice with didstilled water and resuspended in the TCA solution. Each determination was carried out in triplicate. The P_i content is expressed in $mmol.Kg^{-1}$ dry weight. Standard errors of the mean are given.

labile high polymer poly P (1c). In fraction 1b nucleotides may have been present but according to literature (Langen & Liss, 1958, Ehrenberg, 1961) only in very low quantities. Fraction 2 refers to apolar P compounds and fraction 3 to TCA insoluble, probably high molecular weight, poly P. The last fraction 4 consists of e.g. RNA, DNA, nucleotides and possibly phosphoproteins, phosphomannan and sugarphosphates.

The orthophosphate content of the various cells did not differ much. The labile P fraction (1b) differed greatly for Cd resistant and wild type cells. For both fraction 1b and fraction 1c the differences were more pronounced for S. cerevisiae than for S. pombe. There was no difference in the lipid content for two strains of S. cerevisiae. The TCA insoluble poly P fraction was in both S. cerevisiae and S. pombe much smaller than the soluble poly P fraction. There were no significant differences in P content in these fractions between S. cerevisiae wild type cells and Cd resistant cells. However, in the case of S. pombe a significant decrease in Pi content of fraction 3 was found in the Cd resistant cells. In fraction 4 a large difference in P content was found between the wild type cells of S. cerevisiae and the corresponding Cd resistant cells, whereas with the two strains of S. pombe no significant difference was found.

DISCUSSION

During intoxication of wild type cells of S. cerevisiae by Cd^{2+} both a massive loss of cell K^+ and a reduction in viability occur, Gadd and Mowl, 1983, Kessels et al., 1985 A Cd resistant strain of S. cerevisiae Delft 2, obtained by transferring wild type cells to media with increasing Cd concentrations, is able to grow in medium with even 2 mM $CdCl_2$ present. Growth of wild type cells in contrast is retarded by 1 uM $CdCl_2$ in the medium already. Furthermore Cd^{2+} induced loss of cell K^+ is greatly reduced in the Cd resistant cells of S. cerevisiae strain delft 2 (see chapter 9) On comparing Cd resistant cells with wild type cells, Cd uptake is reduced with 50-75% (see chapter 7). This makes it unlikely that the enormous difference in sensitivity towards $CdCl_2$ between wild type and Cd resistant cells of S.

cerevisiae Delft 2 can be ascribed to the reduction in Cd uptake alone.

Efforts to demonstrate the involvement of Cd binding proteins in Cd resistance of S. cerevisiae against 2 mM $CdCl_2$ in the growth medium failed (see chapter 4). On the other hand in the Cd resistant strain of S. pombe, as well as in the wild type strain, we found Cd binding proteins after induction with $CdCl_2$. However, we have good arguments that also in these two cell types the Cd binding proteins do not play an important role in the resistance against cadmium. These findings forced us to search for other possible mechanisms involved in Cd resistance of yeast cells. Because polyphosphate, present in yeast cells is able to act as a trap for cations (Roomans, 1980), it was worth wile to study the distribution of phosphate compounds and their composition in both wild type cells and Cd resistant cells of S. cerevisiae and S. pombe.

We found with both chemical analysis and ^{31}P-NMR spectroscopy that the poly P content of the Cd resistant S. cerevisiae strain Delft 2 was much lower than that of the parent wild type strain. Chemical analysis of the cells revealed approximately a two times higher poly P content of the wild type cells. The NMR data indicate that part of the poly P in the cells are strongly immobilized and do not contribute to the intensity of the poly P peak in the NMR spectra of intact cells. The ratio of the poly P content of the wild type cells and that of the Cd resistant cells as detected by NMR is much higher than that found by means of chemical analysis, see table 1 and also compare Fig. 1 and 2a. Apparently Cd resistant cells not only contain less poly P than the wild type cells but the poly P in the Cd resistent cells also occur partly in an immobilized state. These immobilized poly P become visible in the NMR spectrum on boiling the cells and on increasing the pH of the boiled cell suspension, see Fig. 2. Chemical analysis of S. pombe cells showed a reduction of about 30% in the poly P content of the Cd resistant strain. This is also found by means of ^{31}P NMR spectroscopy indicating that in Cd resistant cells of S. pombe immobilization of poly P does not occur. The distribution between low molecular weight and high molecular weight poly P in the various strains examined, is according to table 1 approximately 1:1. This point, however, is still under examination. In summing up, poly

P contents of the Cd resistant cells of both S. cerevisiae and S. pombe appear to be reduced though more pronounced in S. cerevisiae than in S. pombe.

Besides changes in the amounts of poly P also changes in fraction 4, containing RNA, DNA, nucleotides, phosphomannan and sugarphosphates are found. This is seen only with S. cerevisiae and not with S. pombe, indicating that the changes are not essential for the resistance against Cd^{2+}.

A possible physiological meaning of the observed decrease in poly P in Cd resistant yeast cells is as follows. Yeast cells are able to maintain a low cytosolic Ca^{2+} content (Eilam, 1982). Both an effective extrusion system (Nieuwenhuis, 1981) and accumulation of Ca^{2+} in vacuoles (Eilam, 1985, Ohsumi, 1983, Okorokov et al., 1983) or phosphate-rich cytosolic granules (Roomans, 1980) are involved in the Ca^{2+} homeostasis of the cytosol. Roomans, 1980, furthermore showed that uptake of divalent cations in yeast depends upon the phosphate content of the cells. When, during Cd intoxication Cd^{2+} enters the cells, it may chase Ca^{2+} from the granules and vacuoles and thus lead to an increase in cytoplasmatic Ca^{2+} concentration. An increased Ca^{2+} level in the cytoplasma in turn may provoke loss of cell K^+ by opening K^+ channels. The presence of Ca^{2+}-activated K^+ channels has recently been shown by Gustin et al., 1986. Opening of K^+ channels may lead to a hyperpolarization of the cell membrane, which may result in an increased Cd^{2+} uptake. As a matter of fact, as shown earlier by Kessels et al., 1987, the influx rate of Cd^{2+} is increased during accumulation of Cd^{2+} into the cells of S. cerevisiae. Thus, in our hypothetical model for the Cd intoxication and Cd resistance in yeast, Ca^{2+} in vacuoles or granules plays a crucial role. When less poly P is present in Cd resistant cells, the chance that cytosolic Ca^{2+} homeostasis will be disturbed by Cd^{2} will be smaller because less Ca^{2+} might be available in the storage compartments. Finally it might be hypothesized that the observed protection by Ca^{2+} of yeast cells against Cd^{2+} (Kessels et al., 1985) resides in the prevention of opening the K^+ channels by externally present Ca^{2+} (see also chapters 3 and 9).

Concerning the P-mannan content of the cell wall in relation to the observed agglutination of Cd resistant cells of S. cerevisiae,

we can draw the following conclusion from the ^{31}P-NMR spectra. Since there were no appreciable differences in peak intensities of P-mannan between wild type and Cd resistant cells, agglutination apparently is not due to an increased P-mannan content of the cell wall Furthermore both the wild type- and the Cd resistant- cells of S. pombe did not possess detectable amounts of P-mannan although the resistant cells also displayed a high degree of agglutination. The observed agglutination in both Cd resistant yeast strains therefore must originate from another phenomenon than an increased P-mannan content of the cell wall.

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CHAPTER 6

EFFECT OF Cd ON THE ATPase ACTIVITY IN ISOLATED PLASMAMEMBRANES OF WILD TYPE AND Cd RESISTANT CELLS OF SACCHAROMYCES CEREVISIAE

SUMMARY

Plasmamembranes from wild type and Cd resistant cells of S. cerevisiae strain Delft 2 were isolated. The sensitivity of the ATPase in these membranes towards Cd^{2+} was tested. There only appeared to be a small difference in sensitivity which was not large enough to account for the large difference in sensitivity of the two yeast strains towards Cd^{2+}. Cd^{2+} inhibition of a plasmamembrane preparation from wild type cells of Delft 2 obeyed simple Michaelis Menten kinetics. Ca^{2+} inhibition of the same ATPase preparation, however, rendered a double Hofstee plot indicating the presence of two different enzymes in the preparation or two separate Ca^{2+} binding sites on a single ATPase enzyme. Apparently Cd^{2+} and Ca^{2+} bind to different sites on the enzyme(s).

INTRODUCTION

The way in which Cd^{2+} intoxicates yeast has already been the subject of several studies (Norris and Kelly, 1977 Heldwein et al., 1977, Macara, 1978, Gadd and Mowll, 1983 Kessels et al., 1985, Kessels et al., 1987). Intoxication of the cells by Cd^{2+} is accompanied by two events. Cd^{2+} leads to an efflux of K^+ (Norris & Kelly, 1977, Gadd & Mowll, 1983, Kessels et al., 1985) and Cd^{2+} increases the uptake of both carrier-free ^{45}Ca and ^{109}Cd (Kessels et al., 1987) In this respect Cd^{2+} acts in a similar way like a great number of organic compounds, which share the property of being inhibitors of plasmamembrane ATPase (Pẽna, 1978, Eilam, 1984, Borst-Pauwels et al., 1986, Theuvenet et al., 1986 and Kessels et al., 1987). Three possible models may account for the change in calcium fluxes across the yeast cell membrane (Borst-Pauwels et al , 1985)

According to the first theory Cd^{2+} increases the permeability of the cells for monovalent and divalent cations. This theory accounts for the increased efflux of K^+ from metabolizing cells observed when Cd^{2+} is added (Norris & Kelly, 1977, Gadd & Mowll, 1983, Kessels et al., 1985). In a recent paper Kessels et al., 1987 demonstrated an increased uptake of both

carrier-free ^{45}Ca and ^{109}Cd by S. cerevisiae, strain Delft 2, when 1 mM $CdCl_2$ was present in the medium. These observations are in accordance with the hypothetical increase in cation permeability of the cell membrane.

According to the second theory the increase in ^{45}Ca or ^{109}Cd uptake provoked by Cd^{2+} is caused by hyperpolarization of the cells. Hyperpolarization may be the result of an increase in K^+ permeability of the cell membrane caused by Cd^{2+} The difference with the first theory is that the permeability of the plasmamembrane is selectively increased for K^+ only and not for divalent cations. Also for this second theory Kessels et al., 1987 found indications using the lipophilic cation tetraphenylphosphonium (TPP). The distribution of TPP across the plasma membrane can be regarded as an indicator of the membrane potential. The initial rate of TPP uptake into yeast cells increases by the administration of Cd^{2+} to the medium.

According to the third theory Cd^{2+} acts as an inhibitor of the plasmamembrane ATPase. In that way proton extrusion from the cells is inhibited, resulting in a decrease of the cell pH. Analogous to the situation in Neurospora crassa (Stroobant et al., 1980) in which a Ca^{2+}/H^+ antiport is postulated to be involved in electroge-nic Ca^{2+} release from the cells, we may hypothesize that such a pump is also involved in yeast Ca^{2+} efflux Nieuwenhuis et al., 1981, have shown that in fact a Ca^{2+} pump mechanism exists in yeast, though the nature of this pump system untill now is still not elucidated The driving force of such a pump will be decreased, when the cell is acidified. Then Ca^{2+} extrusion also decreases and the free cytosolic Ca^{2+} concentration is expected to increase. This may lead to opening of K^+ channels in the plasma membrane and concomitant loss of cell K^+. The decreased pumping activity will also lead to an increased accumulation of ^{45}Ca or ^{109}Cd provided that Cd^{2+} is also extruded by the Ca^{2+} pump. This theory is supported by the fact that the cell pH decreases during intoxication of yeast cells by Cd^{2+} (Kessels et al., 1987).

It will be obvious that it is very difficult to discriminate between the theories mentioned above Efforts to distinguish whether changes in membrane permeabilities and/or changes in membrane potentials are the main cause for the increase in ^{45}Ca or

^{109}Cd uptake in yeast did not lead to a clear conclusion (Borst-Pauwels et al, 1986, Kessels et al., 1987).

In this study we determined the sensitivity of isolated plasmamembrane ATPase from both wild type and Cd resistant cells towards Cd^{2+}. As shown by Ahlers & Rosick, 1985, Cd^{2+} inhibits yeast plasma membrane ATPase. According to the third theory, a decrease in Cd^{2+} sensitivity of the ATPase from the Cd resistant cells might provide an important contribution to the observed Cd resistance. If the ATPase is less inhibited, the cytosolic Ca^{2+} concentration will increase less and loss of K^+ may be decreased.

MATERIALS AND METHODS

Organisms and culture conditions. Both the wild type as well as the Cd resistant strain of Saccharomyces cerevisiae Delft 2 were grown on medium A (1% w/v Yeast Extract, 2% w/v Bacto-peptone, 2% w/v glucose, 0.2% w/v $MgCl_2.6H_2O$, 3.46% w/v KH_2PO_4, 0.04% w/v and K_2HPO_4, brought to pH 4.5 with HCl) in 250 ml batch cultures on an orbital shaker at 125 r.p.m. and 30° C. The Cd resistant strain of S. cerevisiae Delft 2 was obtained by culturing wild type cells in the presence of 1 µM $CdCl_2$ and doubling this concentration with each tranfer to new medium. After 48 h of growth, cells were harvested by centrifugation, washed with distilled water and with buffer (0.2 M sucrose, 0.2 M KCl, 5 mM $MgSO_4$, 7 mM Na_2EDTA, 50 mM Imidazol/HCl pH 7.0). Then the cells were resuspended in buffer at a density of about 125 mg dry weight per ml.

Isolation of plasmamembrane ATPase. The cell suspensions in buffer 1 were cooled down to 4° C and kept at that temperature during almost the entire ATPase-isolation procedure. Homogenisation of the cells was achieved in a Braun homogenizer filled with glasspearls of 4.5 mm diameter during 15 min in the case of wild type cells and during 30 min in the case of the Cd resistant cells. The extent of destruction of the cells was estimated microscopically. After several centrifugation steps the resulting membrane fractions were submitted to centrifugation in discontinuous sucrose gradients followed by centrifugation in continuous sucrose

gradients according to Peters and Borst-Pauwels, 1979. After the last sucrose centrifugation step the ATPase containing fractions were diluted and spun down in a Sorvall SS-34 roter at 50000xg for 2h at 4° C. At this stage of membrane isolation two separate methods were followed. In method 1 the membrane pellets were resuspended in 0.2 M sucrose, 200 mM KCl and 50 mM Imidazole/HCl pH 7.0 and stored at -20° C. In method 2 the membrane pellets were submitted to acid precipitation of contamina-ting proteins. The pellets were resuspended in buffer containing 1 mM ATP, 1 mM EDTA, 200 mM KCl and 10 mM Tris brought to pH 7.0 with HCl. Additionally, the suspensions containing the plasmamembrane ATPase were brought to pH 5.2 with diluted acetic acid, instantaneously centrifuged at 7700xg for 1 min (SS-34 Sorvall rotor) and their supernatants immediately brought to pH 7 with 1 N HCl (Dufour and Goffeau, 1978). After centrifugation of the supernatants at 50000xg for 1 h the pellets were resuspended in 0.2 M sucrose, 200 mM KCl and 50 mM Imidazole/HCl pH 7.0 and stored at -20° C. For the acid precipitation step carried out in method 2, Peters and Borst-Pauwels, 1979 showed that partial inactivation of the plasmamembrane ATPase occurred. However, the essential enzymatic properties of the remaining plasmamembrane ATPase activity had not changed (Peters, unpublished results)

Protein content and ATPase assays. The protein content of the obtained ATPase extracts from wild type and Cd resistant Delft 2 cells was determined according to Lowry et al., 1951, using bovine serum albumin as a standard protein. ATPase activities were measured as described by Peters and Borst-Pauwels, 1979. The ATPase incubations were started by addition of 1 ml assay medium to 0.2 ml enzyme solution in order to obtain the initial concentrations of 50 mM Tris/Mes pH 7.0, 200 mM KCl, 4 mM ATP and 4 mM $MgSO_4$. After 60 min at 30° C the reactions were stopped by addition of 1 ml 10% (w/v) trichloroacetic acid. Control determinations were incubated without enzyme. After 60 min first trichloroacetic acid was added and then the enzyme solution. The liberated orthophosphate was determined after the method of Fiske and Subbarow, 1925. All determinations at least were carried out in duplicate. In studies of the effect of azide and diethylstilbestrol 5 mM Mg^{2+} and with vanadate 6 mM Mg^{2+} was applied instead of 4 mM in order to be sure that enough free Mg^{2+} is available for optimal inhibition

(Borst-Pauwels & Peters, 1981). Vanadate-free Na_2H_2ATP was obtained from Boehringer Mannheim, all other chemicals were of analytical grade and purchased from commercial sources.

RESULTS

The sensitivities towards Cd^{2+} and Ca^{2+} of plasmamembrane ATPase, isolated from Delft 2 wild type cells, was tested. Fig. 1 shows the results obtained with a plasmamembrane ATPase preparation that was isolated according to method 1. Half-maximal inhibition ($I_{0.5}$) with $CaCl_2$ was reached at 1.53 mM. At a concentration of 0.4 mM half-maximal inhibition occurred with $CdCl_2$. When the same data were represented as a Hofstee plot, it was obvious that Cd^{2+} inhibition of the plasmamembrane

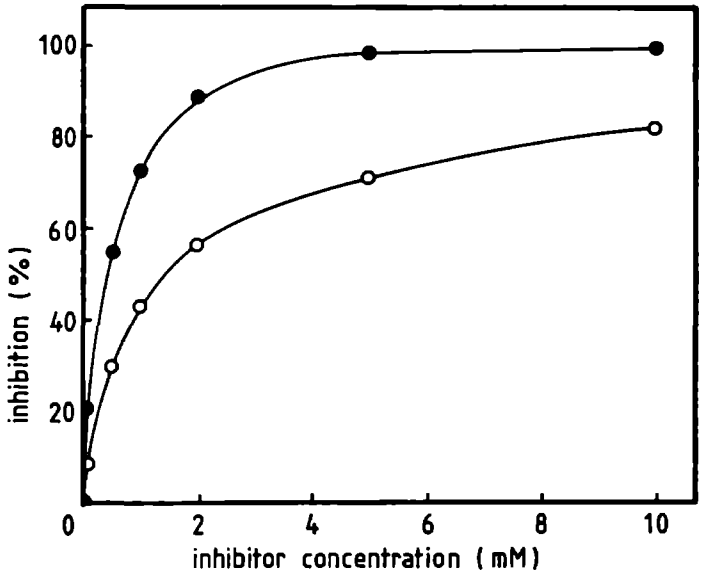

FIG 1. Inhibition of plasmamembrane ATPase from wild type Delft 2 cells by Ca^{2+} (o) and Cd^{2+} (●). Membranes were isolated according to method 1. Inhibition is expressed as percentage of the control. The ATPase assay medium consisted of 50 mM Tris/Mes pH 7.0, 200 mM KCl, 4 mM ATP and 4mM $MgSo_4$.

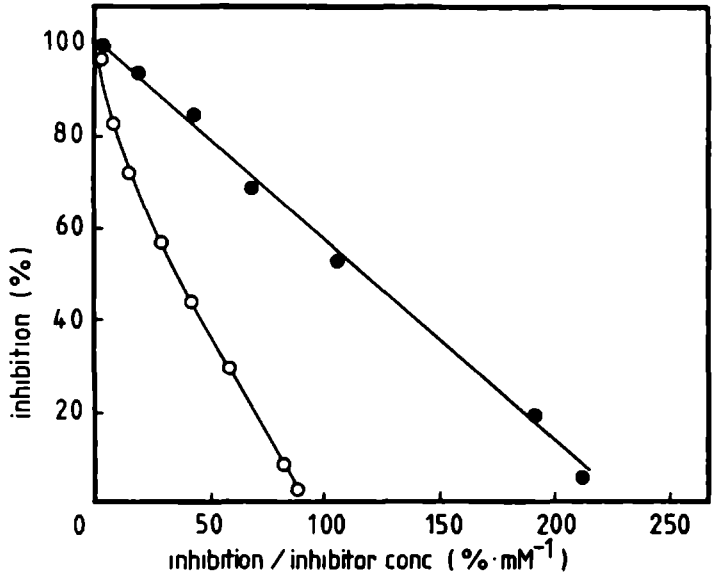

FIG 2. Hofstee plot of the data of Fig. 1. See further Fig. 1.

ATPase displayed Michaelis Menten kinetics with a K_i of 0.44 µM and an I_{max} of 100 %. As shown in Fig. 2, this was not true for inhibition of the ATPase by Ca^{2+}. A concave curve was found. This is typical for the involvement of two different binding sites for the inhibitory cation. The data could be fitted by the sum of two Michaelis Menten equations with corresponding K_i of 0.9 µM and I_{max} of 71 % for the first and K_i=3.1 µM and I_{max}=29 % for the second equation.

After isolation and purification of the membranes according to the acid precipitation method 2, firstly the specific activities, the optimum pH for ATPase activity and the purity of the plasmamembrane ATPase preparations from wild type and Cd resistant cells of S. cerevisiae strain Delft 2 were examined. Fig. 3 shows the results of variation in pH of the assay medium for ATPase activity when 5 mM azide was present. Azide suppresses mitochondrial ATPase activity (Peters and Borst-Pauwels, 1979). The plasmamembrane ATPase preparations from both wild type cells and Cd resistant cells demonstrated an optimum in ATP hydrolysis around pH 6. In order to

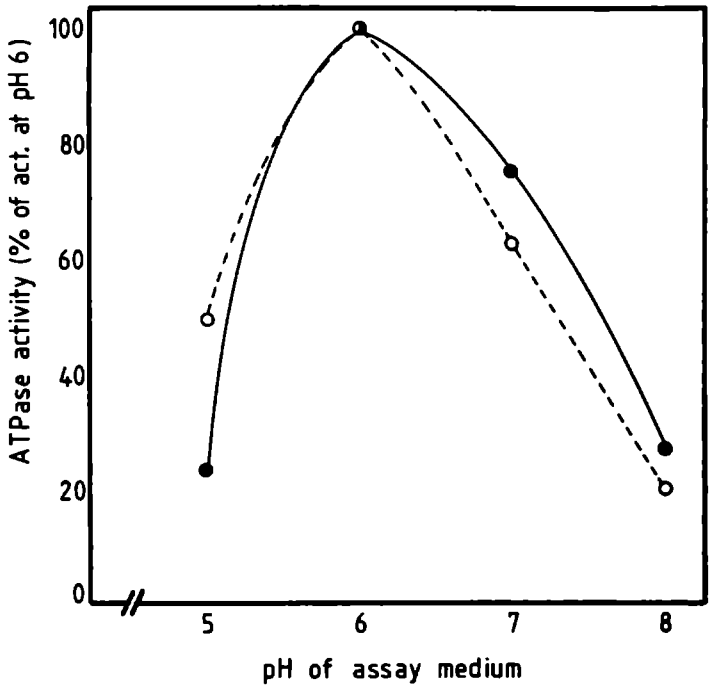

FIG 3. pH dependence of the activity of plasmamembrane ATPase isolated from wild type (o) and Cd resistant cells (●) of S. cerevisiae strain Delft 2. The plasmamembrane preparations were isolated according to method 2. ATPase activity is expressed as percentage of the ATPase activity found at pH 6.0. The pH of the assay medium (see Fig. 1) was set with concentrated HCl or NaOH.

investigate whether the membrane preparations mainly contained plasmamembrane ATPase, we applied two specific inhibitors of the plasmamembrane ATPase, sodiumvanadate and diethylstilbestrol (DES). Their concentrations were 1 µM and 0.17 mM, respectively. Vanadate specifically inhibits plasmamembrane ATPase and DES inhibits both plasmamembrane ATPase and vacuolar ATPase. Inhibition in the presence of DES was 100% with the plasmamembrane ATPase from wild type cells and from Cd resistant cells. Vanadate inhibited the plasmamembrane ATPase from the Cd resistant cells 100% while the plasmamembrane ATPase from wild type cells was inhibited for 97%.

The effect of Cd^{2+} upon the ATPase activity of membrane preparations isolated from both wild type and Cd resistant cells of S. cerevisiae Delft 2, is shown in Fig. 4. The plasmamembrane ATPase

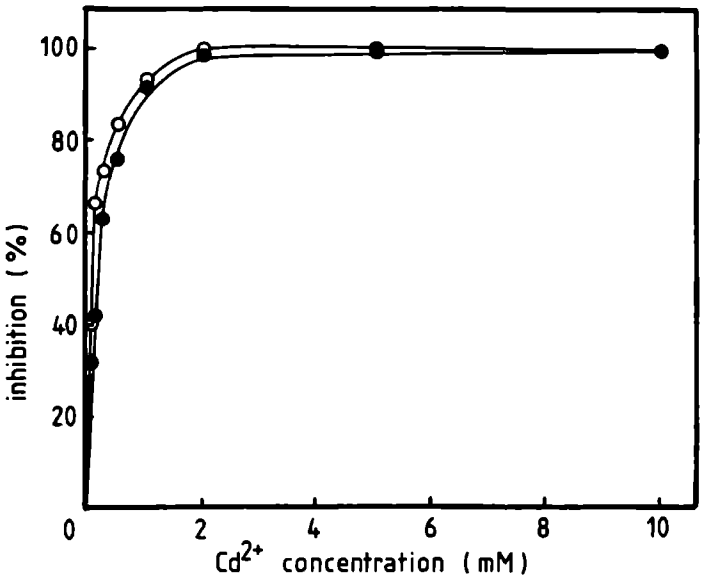

FIG 4. Inhibition by Cd^{2+} of the activities in plasmamembranes isolated from wild type (o) and Cd resistant cells (●) of S. cerevisiae strain Delft 2 according to method 2. Inhibition is expressed as percentage of the control. Except for the Cd concentrations, the ATPase assay medium had the same composition as described for Fig 1.

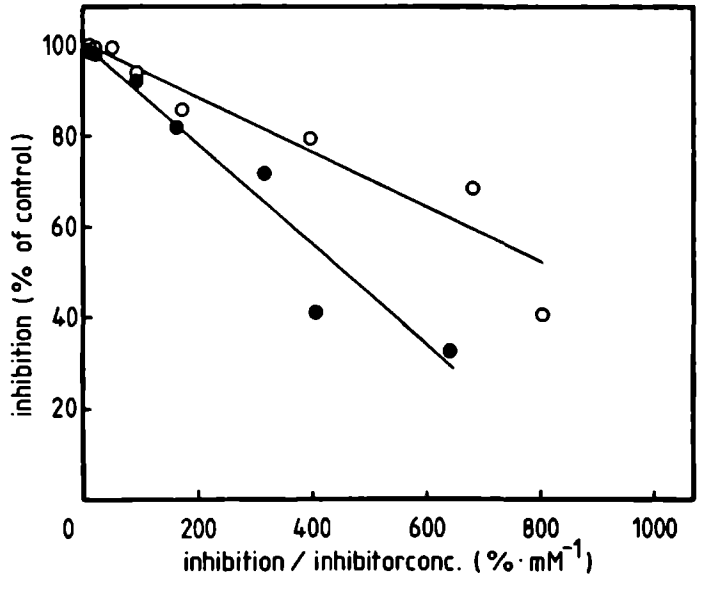

FIG 5. Hofstee plot of the data of Fig. 4. See further Fig. 4.

of the Cd resistant cells appeared to be somewhat less sensitive to Cd^{2+}. The ATPase of both strains was inhibited by Cd^{2+} in a competitive way as is shown in the Hofstee plot of the data presented in Fig. 5.The K_i of Cd^{2+} inhibition for the plasmamembrane ATPase from wild type cells amounted to 63 µM. The corresponding value for the plasmamembrane ATPase from Cd resistant cells was 118 µM.

DISCUSSION

Inhibition of plasmamembrane ATPase by Cd^{2+} and Ca^{2+} apparently are distinct processes. Kinetical analysis of the data leads to the view that Cd^{2+} interacts with only one site in the active centre of the ATPase enzyme. Ahlers & Rosick, 1985, postulated that Cd^{2+} binds to the dissociated form of a sulfhydryl group in the active site of the enzyme. Inhibition of the plasmamembrane ATPase by Ca^{2+} shows a dual character. Therefore Ca^{2+} may interact with two sites on the ATPase. Another explanation for the dual character of Ca^{2+} inhibition may be the presence of two different ATPases with equal affinities for Cd^{2+} but different affinities for Ca^{2+} in the preparation used. At this stage we are not able to discriminate between both possibilities.

Both the optimum pH of 6 as well as the complete inhibition by DES and vanadate found with the plasmamembrane preparations isolated from wild type and Cd resistant cells of strain Delft 2 are characteristics specific for plasmamembrane ATPase. The difference in sensitivity is very small. Cd resistant cells are able to grow in the presence of 2 mM $CdCl_2$, whereas 1 µM $CdCl_2$ already considerably reduces the growth of wild type cells. Therefore it is very unlikely that a decrease in Cd sensitivity of the plasmamembrane ATPase contributes to a major extent to the Cd resistance of Cd resistant cells. Furthermore the difference in sensitivity towards Cd^{2+} found between the two plasmamembrane fractions may be overestimated. The specific activity of the wild type plasmamembrane ATPase preparation is higher (14.1 mmol P_i per gram protein per h in contrast with 8.1 mmol P_i per gram protein per h for the plasmamembrane ATPase preparation from the Cd resistant

cells). This means that in the plasmamembrane ATPase preparation of the Cd resistant cells more contaminating proteins are present. Probably the effective inhibitory Cd^{2+} concentration will be lower because Cd^{2+} partly binds to these contaminating proteins. With the plasmamembrane ATPase preparation from the wild type cells the effective inhibitory Cd^{2+} concentration may be decreased less because less contaminating proteins are present.

The free Cd^{2+} concentration of the cytosol cannot be determined in vivo. Possibly the resistance against Cd^{2+} is due to a more pronounced decrease in cellular free Cd^{2+} in the Cd resistant cells than in the wild type cells. If this is true, inhibition of the plasmamembrane ATPase in Cd resistant cells will be less than in the wild type cells at a given Cd content of the cells. However, until now there are no indications that specific Cd^{2+} binding proteins are present in the Cd resistant cells, see chapter 4. Furthermore cellular polyphosphate, another possible candidate for trapping Cd^{2+} being accumulated into the cells, is present in a lower amount in Cd resistant cells than in the wild type cells, see chapter 5.

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CHAPTER 7

Cd^{2+} UPTAKE AND RELEASE OF CELL K^{+} BY WILD TYPE AND Cd RESISTANT CELLS OF SACCHAROMYCES CEREVISIAE

SUMMARY

Cd resistant cells showed a much smaller release of K^+ than wild type cells. Cd^{2+} uptake into these cells also was considerably decreased The rate of K^+ efflux was not immediately maximal just as was found for wild type cells (see chapter 2). This may be explained on assuming that Cd^{2+} should first enter the cells before giving rise to efflux of K^+. However, at very low cellular Cd^{2+} content K^+ efflux already was relatively large. Furthermore the dependence of K^+ efflux upon the cellular Cd^{2+} content could not be described by a single relationship. With increasing amounts of $CdCl_2$ being initially added to the yeast suspension, an increase in K^+ efflux was found at fixed cellular Cd^{2+} contents. This may indicate that, besides cellular Cd^{2+} also $CdCl_2$ present in the medium contributes to the toxic effect of Cd^{2+}. In the general discussion (chapter 10) further attention will be paid to this latter subject.

INTRODUCTION

Yeast cells need the cations of K, Na, Mg, Ca, and Zn for growth. On the other hand the ions of Cd, Cu, Ag, Hg, Co, Ni, Sn and, in high concentrations also Fe and Zn are toxic to yeast. (White et al., 1951, Maddox & Hough, 1970, Fuhrmann & Rothstein, 1968, Broda, 1972, Norris & Kelly, 1977, Bitton et al., 1984, Joho et al., 1984). The uptake of metal ions comprises two phases (Norris & Kelly, 1977) The first phase consists of a quick and aspecific binding of the cations to negatively charged membrane components located at the cell surface. The second phase consists of energy-dependent intracellular uptake of the metal ions. During uptake of Co^{2+} by yeast cells, an electroneutral 2:1 exchange with cell K^+ was found (Norris & Kelly, 1977) Cd^{2+} uptake by yeast also provokes loss of cell K^+. With Cd^{2+}, however, there is no electroneutral exchange of Cd^{2+} and cell K^+ (Norris & Kelly, 1977, Gadd & Mowll, 1983, Kessels et al., 1985). The molar ratio of K^+ released and Cd^{2+} accumulated by S. cerevisiae in the initial stage of incubation is 22 and seems to be independent

of the Cd concentration (Kessels et al., 1985). Disruption of the cell membrane of part of the cells, according to an all-or-none process, by Cd^{2+} may explain the disproportional loss of cell K^+ during Cd^{2+} uptake (Gadd & Mowll, 1983, Borst-Pauwels et al., 1983, Borst-Pauwels and Theuvenet, 1985).

In chapter 2 it has been shown that efflux of K^+ is not immediate but proceeds with a lag time. A curvilinear relationship was found between the relative rate of net K^+ efflux and the concentration of cellular Cd^{2+}. Therefore it was concluded that the main factor determining the rate of K^+ efflux, is the cellular Cd^{2+} concentration. We now examined whether also a similar relationship exists between the relative rate of K^+ efflux and the cellular Cd^{2+} concentration in Cd resistant cells and wether the resistance towards Cd^{2+} leads to a decrease in the K^+ efflux rate at fixed cellular Cd^{2+} concentrations.

MATERIALS AND METHODS

Organisms and culture conditions. The Cd resistant strain of S. cerevisiae Delft 2 was obtained by repeated transfer of cells to fresh medium with each time the double amount of $CdCl_2$, starting with 1 µM. At 2 mM $CdCl_2$ the $CdCl_2$ content was not increased anymore because precipitation of complexes between medium constituents and $CdCl_2$ occurred above that concentration. Medium A (1% (w/v) Yeast Extract, 2% (w/v) Bacto-Peptone, 2% (w/v) glucose, 0.2% (w/v) $MgCl_2.6H_2O$, 3.4% (w/v) KH_2PO_4, 0.04% (w/v) K_2HPO_4 brought to pH 4.5 with HCl) was used for the development and maintenance of Cd resistant cells as well as for Cd^{2+}-free growth of both the wild type and the Cd resistant cells. The cells were grown in 250 ml batch cultures at 30° C on a rotary shaker with 125 rpm during 48 h. These cultures were inoculated with 0.5 ml cell suspension from a stationary culture. The Cd^{2+}-free cells were harvested by centrifugation, washed twice with distilled water and resuspended in 45 mM Tris/succinate buffer at pH 5.

For Cd^{2+} uptake and K^+ release experiments, the cells (dry weight 6 mg/ml in 45 mM Tris succinate pH 5) were preincubated

in the presence of 3% (w/v) glucose at 25° C for 30 min under anaerobic conditions whereafter $CdCl_2$ was added at appropriate concentrations. For determination of Cd^{2+} uptake, tracer amounts of $^{109}CdCl_2$ (10^5 cpm/ml) were added together with the non-radioactive $CdCl_2$. The radioactivity in samples taken according to the procedure of Roomans et al., 1979, was measured in a liquid scintillation analyser. K^+ release during $CdCl_2$ incubation was determined by flame spectrophotometry according to Kuypers and Roomans, 1979.

Yeast Extract and Bacto-peptone were purchased from Difco Laboratories, ^{45}Ca and ^{109}Cd were purchased from Amersham. All other reagents were reagent grade and obtained from commercial scources.

RESULTS

Fig. 1 shows dose-response curves of $CdCl_2$ intoxication measured as K^+ release with wild type and Cd resistant cells of S. cerevisiae strain Delft 2. The data found after incubating the cells for 90 min at varying $CdCl_2$ concentrations are given. The K^+ release from the wild type cells was much higher than the

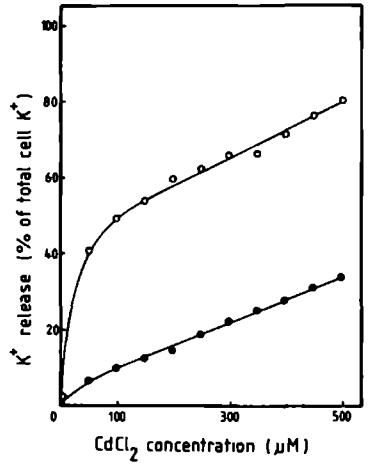

FIG 1. Dose-respons curves of K^+ release and externally applied $CdCl_2$ concentrations found with wild type (○) and Cd resistant (●) cells. The cells were preincubated for 30 min with 3% w/v glucose, Then $CdCl_2$ was added at varying concentrations. K^+ release from the cells, given as the percentage of total cell K^+ initially present, was determined 90 min later. Total K^+ contents of the cells were 625 and 440 mmol per kg dry weight for the wild type and the Cd resistant cells, respectively.

K^+ loss from the Cd resistant cells. In wild type cells, 50 µM $CdCl_2$ already gave rise to a release of approximately 40% of total cell K^+. Under the same conditions only 16% of total cell K^+ was released from the Cd resistant cells. Increasing the Cd^{2+} concentration above 50 µM, led to a further increase in K^+ efflux. This increase was slightly higher for the wild type cells than for the Cd resistant cells.

Fig. 2a and 2b show the results of an experiment in which the release of cell K^+ and Cd^{2+} uptake by Cd resistant cells were simultaneously determined. Comparison with wild type cells (see chapter 2 Fig. 3), showed that the Cd^{2+} uptake was much lower in Cd resistant cells. Just as was found with wild type cells, at relatively low Cd^{2+} concentrations (at 60 and 100 µM), the release of cell K^+ increased initially more than proportionally with the time. At $CdCl_2$ concentrations of 150 µM and higher a rapid but small initial release of cell K^+ was found within the first five min. The release of K^+ from Cd resistant cells was smaller than the K^+ release from wild type cells when incubated

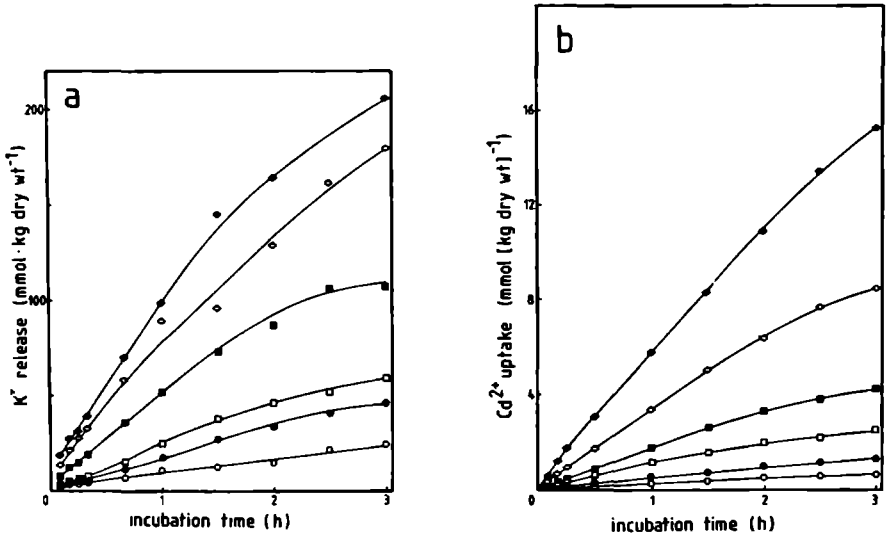

FIG 2. Time course of K^+ release (a) and Cd^{2+} uptake (b) at varying external $CdCl_2$ concentrations for Cd resistant cells. ○ ; 30 µM $CdCl_2$, ● ; 60 µM $CdCl_2$, □ ; 100 µM $CdCl_2$, ■ ; 150 µM $CdCl_2$, ◇ ; 250 µM $CdCl_2$, ◆ ; 500 µM $CdCl_2$. The cells were preincubated with 3% w/v glucose for 30 min. Their total K^+ content was 515 mmol per kg dry weight.

at the same $CdCl_2$ concentrations. At low $CdCl_2$ concentrations the difference in K^+ release from the Cd resistant cells was up to eight times lower. At 500 µM $CdCl_2$, K^+ release from the Cd resistant cells was about three times lower, see also chapter 2. On increasing the $CdCl_2$ concentrations of the medium, there was a concomitant increase in both Cd^{2+} uptake and release of cell K^+.

From the slopes of the curves of Fig. 2a, we obtained net rates of K^+ release. These values were multiplied by the ratio of the total K^+ content of the cells at zero time and the actual K^+ content, see also chapter 2. In this way we corrected for the effect of the decrease in cellular K^+ content upon the net K^+ efflux rate. The resulting relative efflux rates are plotted against the time of incubation in Fig. 3a. Addition of $CdCl_2$ to the medium led to two effects. The first effect consisted of an almost instantaneous increase in the relative rate of K^+

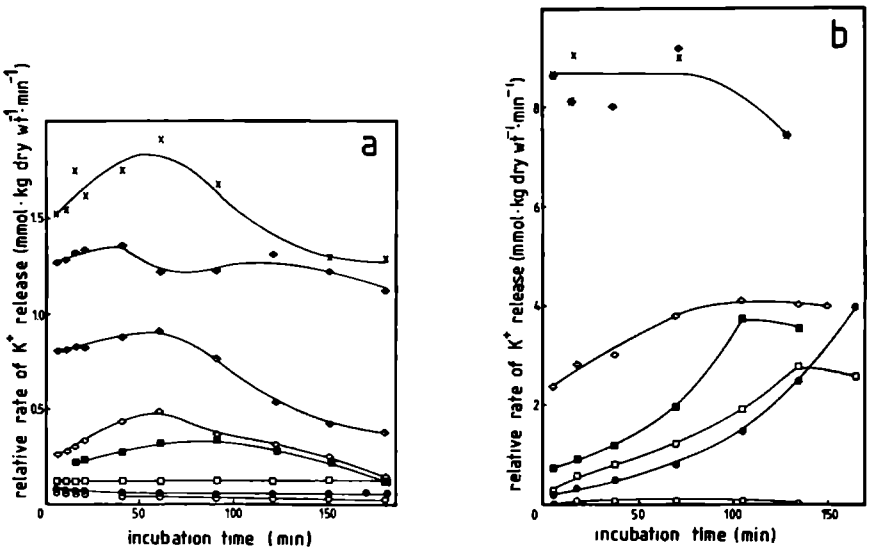

Fig. 3. Time course of the relative rate of K^+ efflux from Cd resistant cells (3a) and wild type cells (3b) during incubation at varying $CdCl_2$ concentrations. The cells were preincubated in the presence of 3% w/v glucose for 30 min. Fig. 3a: ○;0.01mM $CdCl_2$, ●;0.03 mM $CdCl_2$, □;0.06 mM $CdCl_2$, ■;0.1 mM $CdCl_2$, ◇;0.15 mM $CdCl_2$, ◆;0.25 mM $CdCl_2$, ◆;0.5 mM $CdCl_2$. Fig. 3b: ○;0.005 mM $CdCl_2$, ●;0.01 mM $CdCl_2$, □;0.02 mM $CdCl_2$, ■;0.1 mM $CdCl_2$, ◇;0.5 mM $CdCl_2$, ◆, x;1.0 mM $CdCl_2$.

efflux. This almost instantaneous increase in the K^+ efflux rate was followed by a gradual increase in the relative K^+ efflux rate at external $CdCl_2$ concentrations of 60 µM and higher. Similar plots are represented for the relative K^+ efflux rate found in wild type cells, see fig 3b. The data are obtained from chapter 2. Apparently both the almost instantaneous and the subsequent gradual increase in the relative K^+ efflux rate were much larger in wild type cells than in Cd resistant cells. Furthermore in Cd resistant cells the relative net efflux rate reached earlier a maximum whereafter this rate decreased again.

In chapter 2 we showed that, at least by approximation, there was a correlation between the relative net efflux rate of K^+ and the cellular Cd^{2+} content. For the Cd resistant cells it appeared that no single relationship existed between the relative K^+ efflux rate and the Cd^{2+} content, even not by approximation as is shown in Fig. 4. At low cellular Cd^{2+}

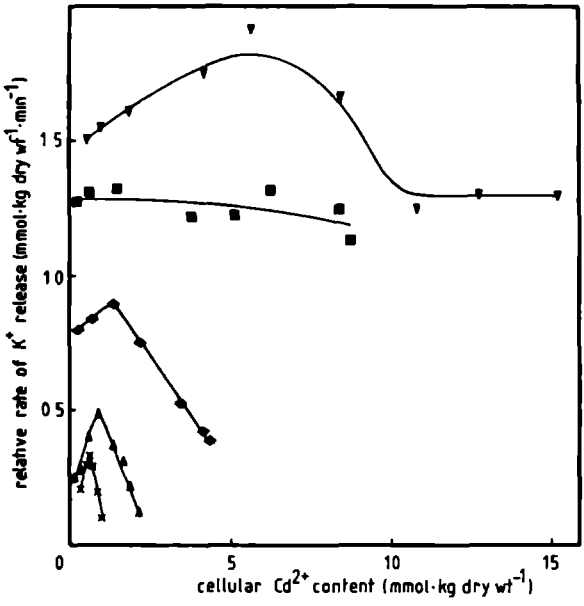

FIG 4. Relation between relative rate of K^+ efflux and cellular Cd^{2+} content during incubation of Cd resistant cells in the presence of 5-500 uM $CdCl_2$ (taken from the results shown in Fig. 3a). ✱ ;0.06 mM $CdCl_2$, ▲ ;0.1 mM $CdCl_2$, ◆ ;0.15 mM $CdCl_2$, ■ ; 0.25 mM $CdCl_2$, ▼ ;0.5 mM $CdCl_2$.

contents the relative K^+ efflux rates increased with the cellular Cd^{2+} content to a maximum and above this concentration a decrease again was found. This was especially apparent when the concentration of $CdCl_2$ initially added to the yeast suspension was low. Furthermore the net efflux rate at a given cellular Cd^{2+} concentration differed greatly at different Cd^{2+} concentrations added to the medium initially.

Fig. 5 shows a plot of the uptake of Cd^{2+} after 5 min of incubation against the concentration of $CdCl_2$ initially added to the cell suspension. Apparently the relation between Cd^{2+}

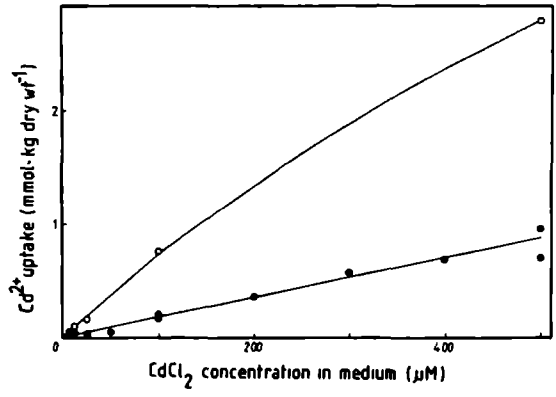

FIG 5. Relationship between Cd^{2+} uptake and the $CdCl_2$ concentration in the medium examined with wild type cells (open symbols) and with Cd resistant cells (closed symbols). Results taken from Chapter 5 Fig. 2a (wild type cells) and from Fig. 2b (Cd resistant cells) in this whapter and from another experiment with Cd resistant cells from which the data were not shown.

uptake and the amount of $CdCl_2$ initially added to the medium, is almost linear. This is found for wild type cells up to a concentration of 0.1 mM and for Cd resistant cells even up to 0.5 mM of the heavy metal. The ratio between the amount of Cd^{2+} uptake after 5 min of incubation found for wild type cells and that found for Cd resistant cells, amounts to 4 for medium concentrations up to 100 μM. At higher medium concentrations the ratio decreases gradually.

There is a large difference in the Cd^{2+} induced release of K^+ from wild type cells and that from Cd resistant cells. This is especially apparent at low doses of $CdCl_2$. In wild type cells Cd^{2+} provoked K^+ efflux is not immediately maximal, see chapter 2 and also Fig. 3b in this chapter. In chapter 2 we hypothesized that this was due to the fact that only internal Cd^{2+} would give rise to release of K^+. This hypothesis was supported by the fact that at relatively low cellular Cd^{2+} contents (below 2 mmol per kg dry wt) a single linear relationship was found between the relative rate of K^+ efflux and the cellular Cd^{2+} content. However, Fig. 3b indicates that almost immediately after the addition of $CdCl_2$ K^+ efflux is provoked. This suggests that part of the K^+ efflux provoked by Cd^{2+} is not due to the intracellular Cd^{2+} but to extracellular Cd^{2+}, a possibility overlooked in chapter 2.

As shown in Fig. 3a, in Cd resistant cells there also seemed to be an almost instantaneous effect of externally added $CdCl_2$ upon K^+ efflux. Subsequently a gradual transient increase in the K^+ efflux rate followed. The latter may be ascribed to interaction of cellular Cd^{2+} with the cells. This view further is supported by the data given in Fig. 4. There was no single relationship between the relative rate of K^+ efflux and the cellular Cd^{2+} content indicating that besides cellular Cd^{2+} also Cd^{2+} of the medium contributes to the toxic effect of $CdCl_2$ upon yeast cells. Apparently Cd resistant cells were less sensitive to both the effect of internal Cd^{2+} and the effect of external $CdCl_2$. For the possibility that still a single mechanism is involved in the intoxication of yeast by Cd^{2+} instead of a dual mechanism by which both external and internal Cd^{2+} are involved, we refer to the general discussion.

This study further shows that uptake of Cd^{2+} in Cd resistant cells is reduced to 25-50% of Cd^{2+} uptake in wild type cells. This reduction certainly may contribute to a decreased Cd^{2+} sensitivity of the Cd resistant yeast strain but it is unlikely that this reduction can account quantitatively for the observed Cd resistance Comparison of Fig. 4 in this chapter and Fig 4 in chapter 2 shows that at a given cellular Cd^{2+} content

K^+ release from Cd resistant cells is smaller than from wild type cells, indicating that the sensitivity towards cellular Cd^{2+} is reduced in the Cd resistant cells. Furthermore the apparent sensitivity towards $CdCl_2$ of the medium seemed to be reduced, as the intercepts in Fig. 3a and 3b differ appreciably.

Both Sr^{2+} and Ca^{2+} uptake in yeast are strongly influenced by the surface potential of the cells, which surface potential in turn depends upon the concentrations of the divalent cations (Borst-Pauwels and Theuvenet, 1984). This results in a less than proportional increase in the influx rate of these cations with increasing substrate cation concentration. Remarkably this is not found for Cd^{2+} uptake. Up to 0.1 mM no deviations from linearity are found between the uptake of Cd^{2+} and the $CdCl_2$ concentrations in the medium for wild type cells. For the Cd resistant cells a linear relation is even found in a wider range of $CdCl_2$ concentrations (0-0.5 mM). It should be stressed, however, that the almost linear relationship between the amount of Cd^{2+} accumulated into the cells within 5 min and the $CdCl_2$ concentration in the medium does not necessarily mean that the initial rates of uptake are linearly related to the $CdCl_2$ concentration in the medium as wel. As has been shown in chapter 3, influx of Cd^{2+} into wild type cells is increased by intracellular Cd^{2+}. There are indications that this also is true for Cd resistant cells (data not shown), which complicates the interpretation of Cd^{2+} uptake kinetics greatly.

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CHAPTER 8

A COMPARATIVE STUDY OF K^+-LOSS FROM A Cd-SENSITIVE AND A Cd-RESISTANT STRAIN OF SACCHAROMYCES CEREVISIAE

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SUMMARY

Cadmium caused less K^+-loss from a Cd-resistant strain of Saccharomyces cerevisiae than from the wild-type. Remarkebly, copper and mercury, as well as a number of organic compounds, structurally unrelated to cadmium, had a similar effect. Contrary to cadmium, however, from the organic compounds the plasmamembrane ATPase inhibitor ethidium, the calmodulin antagonist trifluoperazin, the poly-ene antibiotic nystatin and the cationic detergent cetyltrimethylammonium inhibited the growth of the Cd-resistant strain as effectively as the wild-type. Apparently, the toxicity of these organic compounds was not reduced in the Cd-resistant cells.

The lack of a close connection between the inhibition of cell growth and K^+-loss clearly demonstrates, that investigations on the potential toxicity of compounds to yeasts, measurement of K^+-loss alone is insufficient and should always include tests on viability of the cells.

INTRODUCTION

It is well known that Cd^{2+} causes injury to the cell membrane of yeast, resulting in the release of low-molecular-weight materials and ions from the cells (1-3). The loss of cell K^+ from Saccharomyces cerevisiae for example, is larger than can be accounted for by an electroneutral 2:1 exchange with Cd^+ (1,3,4) which may be considered to be symptomatic of membrane disruption and cell death (1).

Recently we showed that concomitantly to K^+-loss the uptake of Ca^{2+} in S. cerevisiae strain Delft 2 is enhanced by Cd^{2+} (5). The enhancement of opposite fluxes of K^+ and Ca^{2+} (or the analogous divalent cation Sr^{2+}) in yeast is also provoked by a variety of xenobiotics, structurally unrelated to Cd^{2+}. This applies to the inhibitors of the plasmamembrane ATPase Dio-9 (6,7), miconazole (7), diethylstilbestrol (8), ethidium (9-12) and suloctidil (13), the calmodulin antagonists trifluoperazin (9,14,15), compound 48/80 (9), calmidazolium (9) and chlorpromazin (15) which probably also act by virtue of their inhibitory action on the membrane ATPase (9), the polyene antibiotic

nystatin (16) and the membrane permeabilizing agents diethylaminoethyl-dextran (DEAE-dextran)(12) and chitosan (7), as well.

It has been suggested that these xenobiotics, like Cd^{+}, increase the cation permeability of the yeast plasmalemma and provoke an increase in cell Ca^{2+} or Sr^{2+} by making the divalent cations directly accessible to the vacuoles of the cells having a high capacity to accomodate divalent cations (7,9,12).

A number of organic compounds and Cd^{2+} thus share the property of inducing similar ion fluxes in yeast. It may be hypothesized, therefore, that Cd^{2+} and these xenobiotics cause an increase in cation permeability of the plasmamembrane through a common mechanism. In this view, yeast cells rendered resistant to Cd^{2+} might also have become more tolerant to the xenobiotics. This especially could apply to the Cd-resistant strain of S. cerevisiae produced in our laboratory. This strain namely, contrary to Cd-resistant strains described by others (17,18), does not synthesize Cd-binding proteins (Kessels et al., submitted) nor does it show an appreciably decreased Cd^{2+} uptake (Kessels et al., in preparation), indicating that changes in membrane and/or cell wall properties made the cells tolerant to Cd^{2+} and possibly to permeabilizing agents in general. We have now therefore examined whether toxic organic compounds cause less K^{+}-loss from the Cd-resistant strain than from the wild-type and for some, their effect on cell growth is determined as well. In addition we examined the effect of Cu^{2+} and Hg^{2+} upon K^{+}-efflux from the two strains of yeast, two heavy metals, which also provoke K^{+}-loss from normal Cd-sensitive yeast cells (19,20).

MATERIALS AND METHODS

Organism and culture conditions. S. cerevisiae strain Delft 2 was grown in medium A (1%(w/v) Yeast Extract, 2%(w/v) Bacto-Peptone, 2%(w/v) glucose, 0.2%(w/v) $MgCl_2.6H_2O$, 3.46%(w/v) KH_2PO_4, 0.04%(w/v) K_2HPO_4 brought to pH 4.5 with HCl) on an orbital shaker at 125 r.p.m. in 250 ml, 25 ml or 10 ml batch cultures at 30°C.

The Cd-resistant strain of Delft 2 was obtained by growing wild

type cells of Delft 2 in medium A with 1 μM $CdCl_2$ present and doubling this concentration with each transfer to new medium. Inoculation was carried out by addition of 0.05 ml from the stationary grown cultures to 25 ml of fresh medium. 2 mM $CdCl_2$ was the final concentration at which the Cd-resistant cells were grown. Contrary to a Cd-adapted strain of S. cerevisiae described by Macara (21), the Cd-resistant strain obtained by us remained able to grow unarrested in the presence of 2mM $CdCl_2$ even after ten succesive transfers on Cd-free medium.

Inhibition of growth. Because estimation of viability by the conventional spread plate technique was not possible with the Cd-resistant cells, due to a high degree of agglutination, the effect of xenobiotics upon cell growth was determined and used as an indicator for toxicity of the compounds. Heat sterilized medium A was supplemented with appropriate amounts of the different xenobiotics and then filter sterilized. For inoculation, 0.1 ml from a Cd-free stationary phase culture was added to 10 ml culture media in 25 ml Erlenmeyer flasks. After 48 h under culture conditions, growth was determined as the increase in dry weight. Dry weights of twice distilled water washed samples were determined by means of tared aluminium foil cups which were dried to constant weight at 105 °C.

Net potassium fluxes Uptake of K^+ induced by glucose and K^+-efflux provoked by xenobiotics were measured under anaerobic conditions at 25 °C with a K^+-sensitive glass electrode and registered continueously by means of a recorder. Stationary phase cells were harvested by centrifugation, washed twice with distilled water and resuspended in buffer (20 mM morpholine-ethanesulfonic acid (MES) brought to pH 6.0 with triethanolamine (TEA)) at a density of 100 mg wet weight per ml. From this dense suspension of cells further dilutions were made in the buffer for measurement of K^+ fluxes. Uptake of K^+ was measured in buffer to which an appropriate amount of K^+ was added and initiated by the addition of 3%(w/v) glucose to the cell suspension. For measurement of K^+-loss the cells were diluted in the buffer provided with 3%(w/v) glucose and preincubated for 10 min. Subsequently the xenobiotics were added and K^+-loss was followed.

Chemicals Trifluoperazin, compound 48/80, chlorpromazin,

nystatin and amphotericin B were purchased from Sigma Chemie, Taufkirchen (FRG), calmidazolium from Janssen Life Sciences Products, Beers (Belgium), ethidium bromide from Boehringer, Mannheim (FRG) and DEAE-dextran from Pharmacia, Uppsala (Sweden). Octylguanidine was a gift from Dr. A. Peña (Mexico). Miconazole and suloctidil were gifts from Professor A. Goffeau (Louvain-la-Neuve, Belgium). Dio-9 was a gift from Gist-Brocades at Delft (The Netherlands). 1 mg/ml Dio-9 gave an absorbance of 2.0 at 303 nm after correction for the absorbance at 400 nm. All other chemicals were reagent grade or analytical grade and obtained from commercial sources.

Miconazole was dissolved in dimethylsulfoxyde; nystatin in propane-1,2-diol; Dio-9, compound 48/80, calmidazolium, diethylstilbestrol, octylguanidine and suloctidil in ethanol. In the experiments the ultimate concentration of the solvent was 1%(v/v). The organic compounds as well as chloride salts of the heavy metals used in this study were added to the yeast suspensions from concentrated aquous solutions.

RESULTS AND DISCUSSION

Fig. 1 shows the time course of K^+-loss from wild-type and Cd-resistant yeast cells provoked by different classes of xenobiotics. As was expexted for Cd^{2+}, the heavy metal caused less K^+-loss from the Cd-resistant cells than from the wild-type. This was also found with the heavy metals Cu^{2+} and Hg^{2+}, both provoking a larger loss of cell K^+ from either strain than Cd^{2+}.

The inhibitors of the plasmamembrane ATPase of yeast, ethidium, suloctidil, Dio-9, diethylstilbestrol and miconazol and also octylguanidine, which inhibits the membrane ATPase of the related fungus Neurospora crassa (22) also caused less K^+-loss in the resistant strain. The same phenomenon was also observed with the calmodulin antagonists trifluoperazin, compound 48/80, calmidazolium and chlorpromazin, for which we have recently shown that they also effectively inhibit the membrane ATPase of yeast (9).

The lower panel of Fig. 1 shows the effect of the polyene antibiotics nystatin an amphotericin B, the detergents

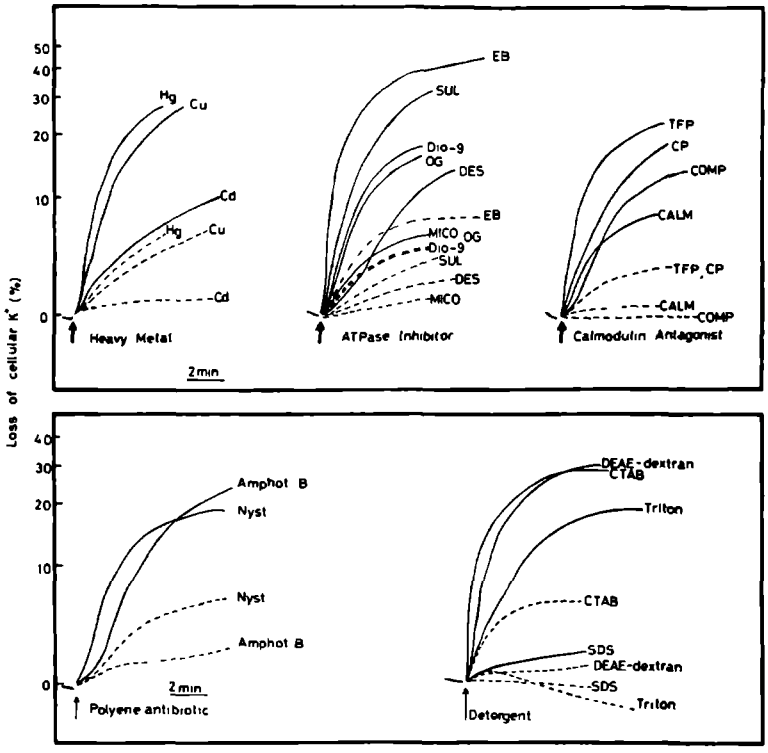

Fig 1: Time course of the K^+-loss provoked by heavy metals and organic xenobiotics in wild-type (——) and Cd-resistant (----) yeast cells. Addition of xenobiotics to yeast suspensions containing 5 mg wet weight of cells per ml, is indicated by the arrow. The final concentration of heavy metals was 0.5 mM, of ethidium (EB) 0.3 mM, suloctidil (SUL) 2 ug/Ml, Dio-9 1.7 ug/ml, octylguanidine (OG) 100 u6/ml, diethylstilbestrol (DES) 100 µM, miconazole (MICO) 1 µg/ml, trifluoperazin (TFP) 31 µM, chlorpromazin (CP) 50 µM, compound 48/80 (COMP) 0.25 µg/ml, calmidazolium (CALM) 0.25 µg/ml, nystatin (Nyst) 5µg/ml, amphotericin B (Ampho. B) 12.3 µg/ml, cetyltrimethyl- ammoniumbromide (CTAB) 25 µM, Triton X-100 0.05% (v/v), sodiumdodecyl- sulphate (SDS) 0.05% (v/v) and DEAE-dextran 0.5 mg/ml. Maximal K^+-loss (100%) was determined by the addition of 0.1 mM CTAB to the cells after each experiment and amounted to 0.83±0.01 mM for either strain of Delft 2 (corresponding to 553±7 moles K^+ per kg dry weight of cells).

cetyltrimethylammonium, sodiumdodecylsulphate and Triton X-100 and also the polybase DEAE-dextran. These compounds caused less K^+-loss from the Cd-resistant strain, as well.

As is shown in Fig. 2 there was a great difference in sensitivity of the two types of cells with respect to cell growth just as was found for K^+-loss. While even at 2 mM $CdCl_2$

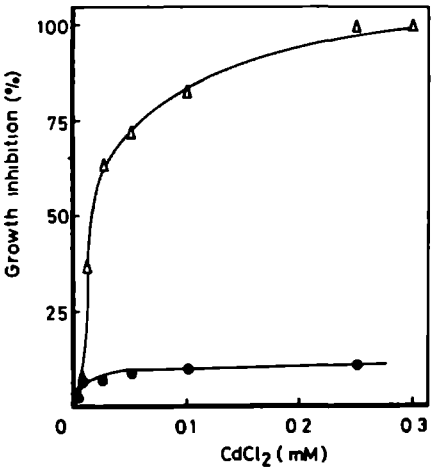

Fig 2. Concentration-dependent inhibition of yeast growth by Cd^{2+}; Δ, wild-type cells; ●, Cd-resistant cells.

the resistant cells were still able to grow almost unarrested (data not shown), 0.2 mM $CdCl_2$ nearly completely inhibited the growth of wild-type cells.

From each class of xenobiotics we have used in Fig. 1, also one was chosen to examine its effect on the growth of the strains. The concentration-dependent inhibition of the growth of the yeast cells by the polyene antibiotic nystatin, the cationic detergent cetyltrimethylammonium, the ATPase inhibitor ethidium and the calmodulin antagonist trifluoperazin is shown in Fig. 3. Although these compounds, just as Cd^{2+}, caused less K^+-loss from the Cd-resistant cells than from the wild-type cells, they inhibited the growth of both strains equally. The growth of both strains was completely arrested at the higher concentrations of nystatin, cetyltrimethylammonium and trifluoperazin. With ethidium only a partial inhibition of growth could be attained (about 65% in the wild-type and about 50% in the Cd-resistant strain). Ethidium bromide, however, also is an extremely effective cytoplasmic mutagen in yeast, producing mutants (23). It thus is possible that during the relative long time interval of the growth experiment the ethidium tolerant mutants were produced in the batch cultures of each yeast.

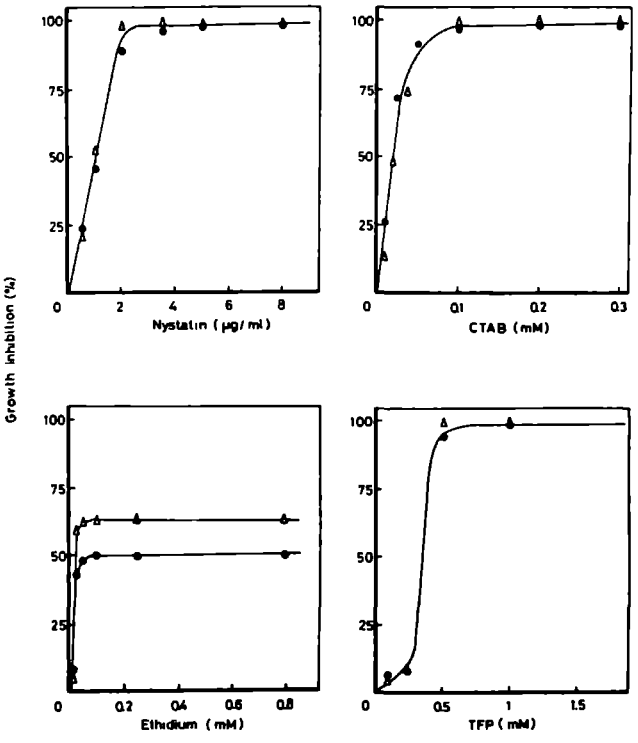

Fig 3. Concentration-dependent inhibition of yeast growth by four organic xenobiotics, see also legend to Fig.2.

Apparently the toxicity of organic xenobiotics to the Cd-resistant strain was accompanied by much less K^+-loss than from the wild-type cells. It should be realized, however, that the organic xenobiotics as well as the heavy metals may provoke K^+-loss from yeast in an all-or-none process. Each cell has according to this view a certain treshold concentration, which if exceeded, results in the destruction of the permeability barrier for K^+ and as a consequence, in an almost complete loss of K^+ from the affected cells. Kuypers and Roomans(24), for example, showed that the increased K^+ release from yeast exposed to increasing mercury levels was due to an increase in the number of cells showing an all-or-none response. We have shown that the calmodulin antagonists trifluoperazin, calmidazolium and compound 48/80 (25)

and the inhibitors of the plasmamembrane ATPase ethidium, Dio-9, diethylstilbestrol, miconazole and suloctidil (26,27) and also CTAB and DEAE-dextran (unpublished) provoke an all-or-none K^+ efflux from yeast cells, as well. This might also apply to the K^+-losses observed in the present study. As the organic xenobiotics arrested the growth of both strains to the same extent, possibly in either strain the same proportion of the cells completely released cell K^+ in an all-or-none process. Uptake of this K^+ by the remainder part of unaffected cells in the suspension would then lead to an underestimation of the proportion of cells that released cell K^+ completely. Taking also in consideration that the mean K^+ content of the cells was approximately the same for both strains (about 550 mmoles/kg dry weight), it may be hypothesized that the smaller net K^+ losses provoked by organic xenobiotics in Cd-resistant cells were due to a more efficient K^+ accumulation in Cd-resistant cells than in the wild-type. An indication that this might be true, is given in Fig. 4, where is shown that Cd-resistant cells reduced the K^+ concentration of the medium to a significantly lower level than the Cd-sensitive cells. In this experiment a relatively high K^+ concentration was added to the yeast suspension to assure V_{max} conditions for the K^+ uptake system (28). Uptake of K^+ was initiated by the addition of glucose to the yeast suspensions. Neither the lag-time nor the maximum rate of K^+ uptake attained

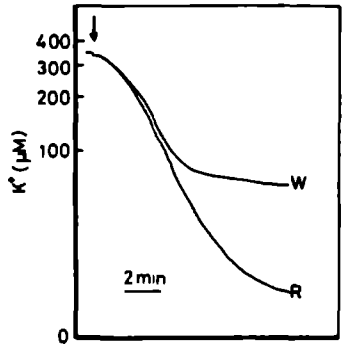

Fig 4. Net K^+uptake by wild-type (W) and Cd-resistant (R) yeast cells induced by the addition of glucose (3% w/v) indicated by the arrow. The suspension contained 10 mg wet weight of yeast per ml.

differed in the two strains, indicating that the more efficient uptake of K^+ in the Cd-resistant cells can not be ascribed to a higher V_{max} for K^+ uptake, but was due to a decreased efflux rate of intracellular K^+ to the medium. The greater K^+ accumulation in Cd-resistant cells might be responsible for the smaller net K^+-losses consistently observed in these cells.

According to the view developed above it should be expected that under conditions that uptake of K^+ becomes quantitatively less important, i.e. at lower cell densities, the large differences in K^+-loss provoked in the two strains by xenobiotics would diminish. The data in Fig. 5 agree with that notion. The percentual K^+-loss provoked by the organic compounds was more increased in the Cd-resistant strain than in the wild-type on reducing the cell density tenfold. For comparison, also the effect of Cd^{2+} was determined. The K^+-loss caused by the heavy metal, however, remained the same, indicating that Cd^{2+} inhibited the uptake of K^+ in both strains strongly preventing reentry of lost K^+.

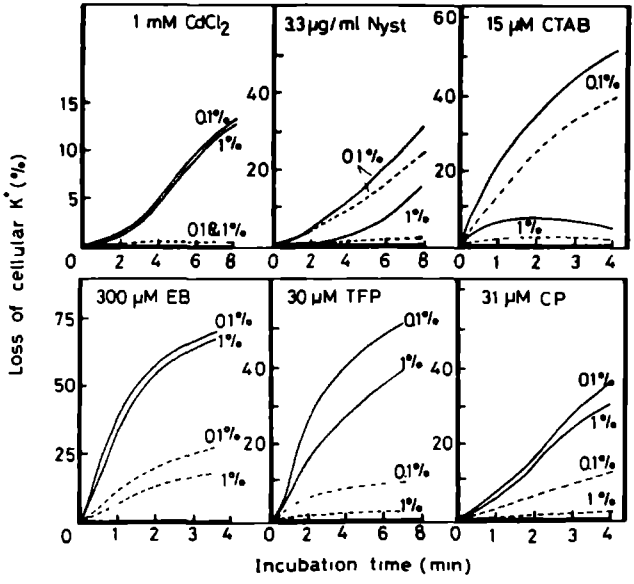

Fig 5. Comparison of the time course of the K^+-loss provoked by Cd^{2+} and organic xenobiotics found at cell densities of 1 and 0.1% (wet weight/volume), see also legend to Fig.1.

In summing up, the work presented here shows that although a large number of organic compounds caused less K^+-loss from the Cd-resistant strain than from the wild-type, they were not less toxic to the Cd-resistant strain. The difference in K^+-loss can be explained on assuming that the K^+-loss was an all-or-none process and it is argued that the differences in K^+ efflux rates between still unaffected cells in either strain might be responsible for the larger losses of K^+ from the wild-type than from the Cd-resistant strain. Our present findings warn against the use of K^+-loss as a sole indicator for the toxicity of xenobiotics to yeasts.

ACKNOWLEDGEMENTS

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CHAPTER 9

EFFECTS OF Cd^{2+} UPON GROWTH, CELL K^{+} AND VIABILITY OF WILD TYPE AND Cd RESISTANT CELLS OF SACCHAROMYCES CEREVISIAE

SUMMARY

Cd resistant cells of Saccharomyces cerevisiae strain Delft 2 were exposed to $CdCl_2$. Inhibition of growth by $CdCl_2$ was studied in nutrient medium. The release of cell K^+ and changes in viability of both wild type and Cd resistant cells were studied in Tris/succinate assay medium supplied with glucose and varying amounts of $CdCl_2$. The Cd^{2+} induced release of cell K^+ from both wild type and Cd resistant cells was partly prevented by Ca^{2+}. Viability was less pronounced influenced by Ca^{2+}.

The application of K^+ release or changes in viability as a measure for $CdCl_2$ intoxication of yeast is discussed. The percentage of K^+ released from the cells in the presence of Cd^{2+} is lower than the percentage of cells that cannot grow anymore. This indicates that efflux of K^+ cannot be used as a quantitative measure for Cd^{2+} intoxication.

The Cd resistant cells were much smaller than the parent wild type cells and showed a high degree of agglutination.

INTRODUCTION

There are two ways by which yeast strains can acquire a decreased sensitivity towards cadmium. One way is by means of physiological adaptation (Nakamura and Ashida, 1959, Macara, 1978 and Minney and Quirk, 1985) After repeated subculturing in Cd^{2+} containing media a strain is obtained that can grow at high concentrations of $CdCl_2$ In the case of adaptation the acquired resistance against Cd^{2+} will be lost again after repeated culturing of the Cd^{2+} adapted cells in Cd^{2+} free medium. The other way comprises a decreased Cd^{2+} sensitivity obtained by mutation and selection. These Cd resistant yeast strains also can be obtained by repeated subculturing in Cd^{2+} containing media Mutagenesis by U.V. irradiation, in combination with selection on Cd^{2+} containing media, may stimulate the generation of Cd resistant yeast strains. Cd resistance, evoked by mutation and selection, does not disappear after several transfers to Cd^{2+} free media (Nakamura, 1963, Tohayama and Murayama 1977). Cd adapted yeast strains usually can tolerate higher

Cd^{2+} concentrations in the nutrient medium than Cd resistant yeast strains The Cd resistant strain of Saccharomyces cerevisiae Delft 2 used in this study is able to grow in nutrient medium with 2 mM $CdCl_2$ present. After ten transfers in Cd^{2+} free medium, no decrease in Cd^{2+} sensitivity occurs. Apparently the Cd resistant yeast strain used in this study, is of the mutation and selection type. Nevertheless this strain is displaying resistance against high medium concentrations of $CdCl_2$.

Generally Cd^{2+} uptake is reduced in Cd adapted and Cd resistant yeast strains (Joho et al., 1985, Joho et al., 1986). Additionally, Cd binding proteins and polyphosphates sometimes appear to be involved (Nakamura, 1965, Joho et al., 1985) in Cd adaptation or Cd resistance. Recent results obtained with the Cd resistant strain Delft 2 learned that neither synthesis of Cd binding proteins nor increased polyphosphate content of the cells contribute to the extreme Cd resistance of this strain. The mechanisms of Cd toxicity and Cd resistance in yeast are still poorly understood. Even within this genetically closely related species of yeast the divergence in Cd sensitivity and the cellular response to Cd^{2+} are too large to justify a general hypothesis for Cd toxicity and Cd resistance.

In this study comparison is made between a Cd resistant strain of S. cerevisiae Delft 2 and its wild type parent strain. We examined growth inhibition, release of cell K^+ and decrease in cell viability during Cd^{2+} intoxication and the effect of Ca^{2+} upon these parameters. As shown in chapter 2, Ca^{2+} protects the wild type cells against Cd^{2+}. Both the decrease in growth and loss of cell K^+ are much less in the presence of Ca^{2+} than in its absence. We now examined wether Ca^{2+} also affects these parameters in Cd resistant cells. In addition it was examined wether Ca^{2+} also influences the viability of the cells during Cd^{2+} incubation A comparison was made between K^+ loss and loss of viability as indicators of Cd^{2+} toxicity.

MATERIALS AND METHODS

The Cd resistant strain of S. cerevisiae strain Delft 2 was obtained by repeated subculturing in medium with increasing

$CdCl_2$ concentrations. 50 ml Erlenmeyer flasks containing 25 ml medium A (1% w/v Yeast Extract, 2% w/v Bacto-Peptone, 2% w/v glucose, 0 2% w/v $MgCl_2.6H_2O$, 3.46% w/v KH_2PO_4, 0 04% w/v K_2HPO_4, brought to pH 4.5 with HCl) were used with 1 uM $CdCl_2$ as the first and 2 mM $CdCl_2$ as the final concentration. The $CdCl_2$ concentration was increased twice in each successive step. After inoculation with 100 ul from the preceeding $CdCl_2$ containing stationary phase culture, the batch cultures were incubated on an orbital shaker at 125 rpm and at 30° C.

For growth experiments 250 ml medium A was inoculated with 0.5 ml cellsuspension from a stationary phase culture. Samples, taken at appropriate times during growth and ranging from 5 to 20 ml in volume, were centrifuged, washed twice with 1 ml distilled water, resuspended in 1 ml distilled water and dried to constant weight at 105° C in aluminium trays.

For K^+ release and cell viability experiments, stationary Cd-free cells were harvested by centrifugation, washed twice with distilled water and resuspended in 45 mM Tris/succinate buffer at pH 5.0. Cells were preincubated for 30 min in the presence of 3% w/v glucose at 25° C under anaerobic conditions and $CdCl_2$ was added at appropriate concentrations. K^+ release during $CdCl_2$ incubation was determined by flame spectrophotometry of the supernatants after centrifugation according to Kuypers & Roomans, 1979. Viabilities were determined by means of the spread plate technique using solid YNB medium (0.67% w/v Yeast Nitrogen Base, 1% w/v glucose and 2% w/v Bacto Agar) for growth and distilled water as the diluent. When the Petri dishes had been incubated at 30° C for 48 h, the numbers of colonies were counted. The number of cells transferred to the dishes was of the order of magnitude of 300. Cd^{2+} uptake was determined as described in chapter 2 for wild type cells using ^{109}Cd as the radioactive tracer.

Yeast Extract, Bacto-Peptone and Bacto-Agar were purchased from Difco. All other chemicals were reagent grade and obtained from commercial sources.

The Cd resistant strain of S. cerevisiae Delft 2 was obtained by repeated subculturing in medium with increasing Cd^{2+} concentrations. Above 2 mM $CdCl_2$, complexes between cadmium and medium constituents precipitated, even when medium and the $CdCl_2$ stock solution were heat sterilized separately. For that reason 2 mM $CdCl_2$ in medium A became the highest $CdCl_2$ concentration applied for maintenance of the Cd resistant strain.

Fig. 1 shows photographs of wild type (1a) and Cd resistant (1b) cells of S. cerevisiae strain Delft 2. The Cd resistant cells were about 50% smaller in size than the wild type cells. There was a high degree of agglutination detected in the cell suspensions of the Cd resistant cells. Attempts to reduce this agglutination by means of vigorous shaking, sonification, addition of the detergents Tween 80 and Saponine, or addition of ethylenediaminetetraacetate (EDTA), failed. The morphological characteristics of the Cd resistant cells did not depend upon the presence of $CdCl_2$ during growth. In the

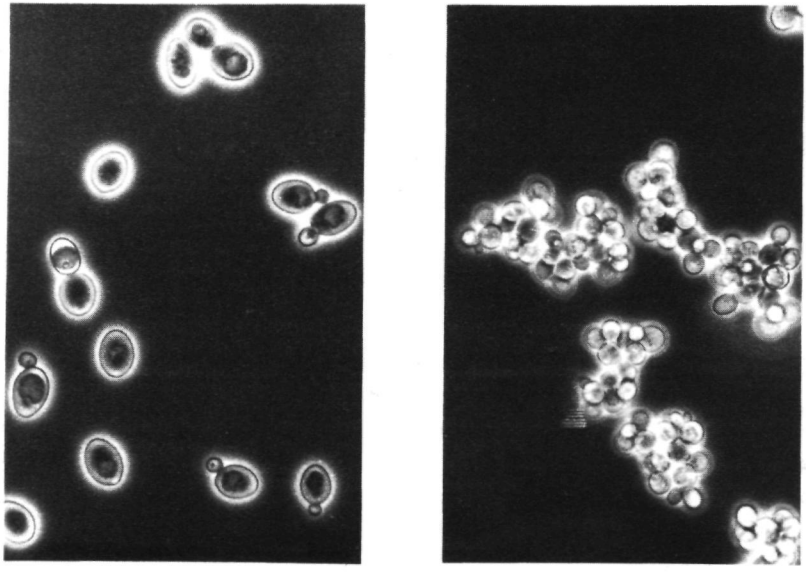

FIG 1. Photographs of wild type (a) and Cd resistant (b) cells of Saccharomyces cerevisiae strain Delft 2. A phase contrast microscope was used with magnification 400x.

presence of 1 mM externally added $CdCl_2$, wild type cells also exhibited agglutination but to a much lower extent than was found with the Cd resistant cells even in the absence of added $CdCl_2$.

Growth of the Cd resistant strain Delft 2 was studied in the presence of 0, 0.1 and 1 mM $CdCl_2$ in the nutrient medium, see Fig. 2a. In comparison with growth of wild type cells under the same conditions, (see chapter 2 Fig. 1), the growth rate of the Cd resistant cells in the absence of $CdCl_2$ was lower. The Cd resistant cells appeared to have reached the stationary phase after about 25 h of growth while the wild type cells already had reached that phase after 15 h. The yeast yield, measured as dry weight per volume, in the Cd free stationary cultures was highest for the wild type strain.

Addition of 0.1 mM $CdCl_2$ to the nutrient medium inhibited the growth of the Cd resistant cells only to a small extent. Growth of the wild type cells under that condition was strongly inhibited (see chapter 2 Fig. 1). When the $CdCl_2$ concentration of the nutrient medium was brought to 1 mM, a small but significant

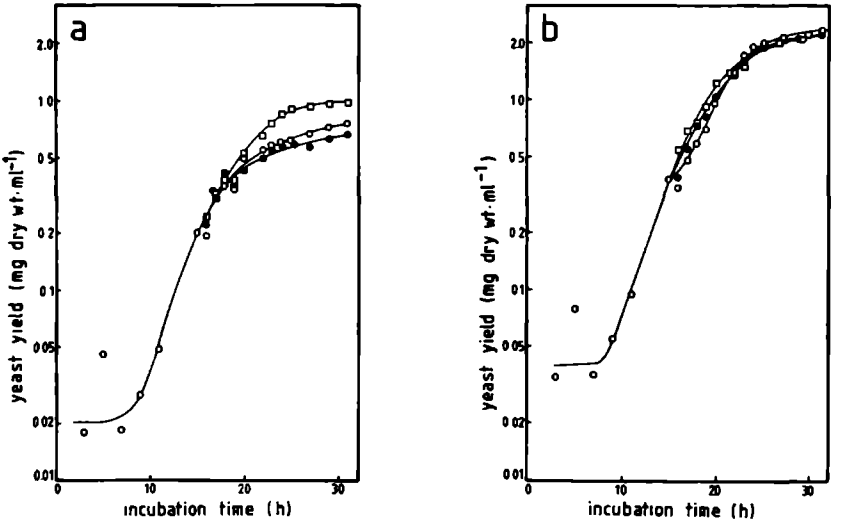

FIG 2. Effect of $CdCl_2$ on the growth of the Cd resistant strain of Delft 2 in the absence (2 a) and in the presence (2b) of 1 mM $CaCl_2$. ○, no $CdCl_2$; ●, 0.1 mM $CdCl_2$; □, 1 mM $CdCl_2$. In all cases $CdCl_2$ was added 15 h after inoculation.

stimulation of growth occurred. Growth of the wild type cells, in contrast, was almost completely arrested.

Growth of the Cd resistant cells appeared to be stimulated by the presence of Ca^{2+} in the nutrient medium. This was detected as an increased yeast yield of stationary phase cultures (Fig. 2b). In the presence of 1 mM $CaCl_2$ addition of 0.1 or 1 mM $CdCl_2$ to the nutrient medium initially increased growth a bit. The yeast yield after 30 h, however, did not significantly differ from that of the control without $CdCl_2$.

Fig. 3 shows the results of a study on the protective effect of calcium upon cadmium intoxication of yeast. We determined the effect of Ca^{2+} upon the Cd^{2+} induced loss of cell K^+ (Fig. 3a) and the decrease in viability of the cells (Fig. 3b) as well. Wild type and Cd resistant cells were incubated anaerobically in 45 mM Tris/succinate buffer pH 5.0, provided with 3% w/v glucose, in the presence of 0.1 mM $CdCl_2$ alone or with both 0.1 mM $CdCl_2$ and 0.1 mM $CaCl_2$ present. 0.1 mM $CdCl_2$ provoked a rapid loss of cell K^+ from the wild type cells, see Fig. 3a.

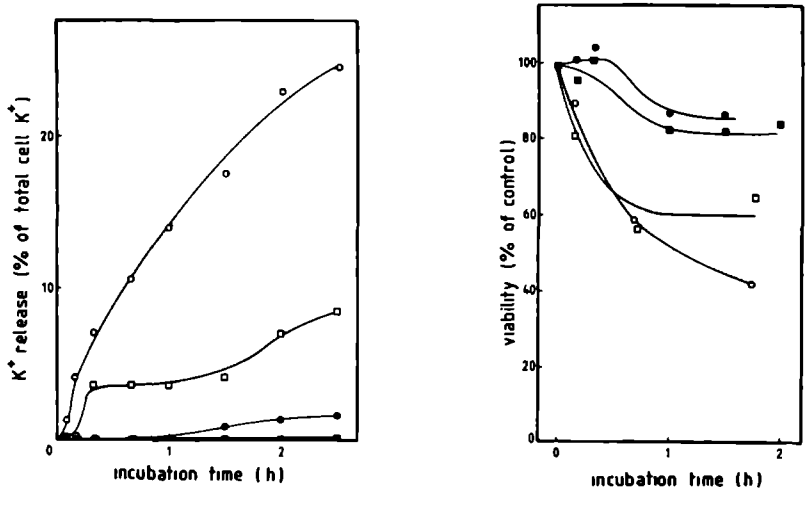

FIG 3. Effect of $CaCl_2$ on K^+ release (3a) and viability (3b) during $CdCl_2$ intoxication of wild type (open symbols) and Cd resistant cells (closed symbols) of strain Delft 2. ○, ●, incubation in the presence of 0.1 mM $CdCl_2$. □; ■, incubation in the presence of both 0.1 mM $CdCl_2$ and $CaCl_2$.

The rate of K^+ release was not immediately maximal. The K^+ loss showed a lag period of a few minutes. The K^+ release amounted to 23% of the total cell K^+ after 2 h. When both 0.1 mM $CdCl_2$ and 0.1 mM $CaCl_2$ were present in the medium, the release of K^+ from the wild type cells showed a lag time of 10 min whereafter a sudden release of K^+ followed. The subsequent release of K^+ was low. After 2 h the K^+ release amounted to 6% of the total cell K^+. 0.1 mM $CdCl_2$ could provoke only a minor K^+ release from the Cd resistant cells (1% of total cell K^+ after 2 h of incubation) and in the presence of $CaCl_2$ together with 0.1 mM $CdCl_2$ no release of K^+ could be detected. There was a lag in the absence of added $CaCl_2$ of about 45 min. Thus 0.1 mM $CdCl_2$ caused a much higher release of cell K^+ from wild type cells than from Cd resistant cells (see also chapter 2) and the presence of $CaCl_2$ reduced the extent of K^+ release provoked by $CdCl_2$ from both wild type and Cd resistant cells.

Under the same conditions as K^+ release was studied, viability of wild type and Cd resistant cells was determined. In the presence of 0.1 mM $CdCl_2$ a large decrease in viability was found with the wild type cells (viability was 43% after 2 h of incubation, see Fig. 3b). When 0.1 mM $CaCl_2$ was added together with $CdCl_2$, the decrease in viability was lower (viability was about 60% after 2 h of incubation). The viability of the Cd resistant cells was slightly increased during the first 20 min of incubation in the presence of Cd^{2+}. After that time a reduction in viability had occurred. After 2 h of incubation in the presence of 0.1 mM $CdCl_2$ the viability of the Cd resistant cells dropped to about 88%. There was only a small effect of 0.1 mM $CaCl_2$ upon the $CdCl_2$ induced decrease in viability of the Cd resistant cells. Remarkably, $CaCl_2$ seemed to intensify the $CdCl_2$ induced decrease in viability.

Fig. 4a and b show the time courses of K^+ release and changes in viability provoked by $CdCl_2$ added at varying concentrations. Even in the absence of $CdCl_2$ a release of cell K^+ from the wild type cells was found. Therefore we substracted the amounts of K^+ being lost in the absence of $CdCl_2$ from those being lost in the presence of $CdCl_2$. The presence of 5 µM $CdCl_2$ did not lead to an increase in the K^+ loss, see Fig.

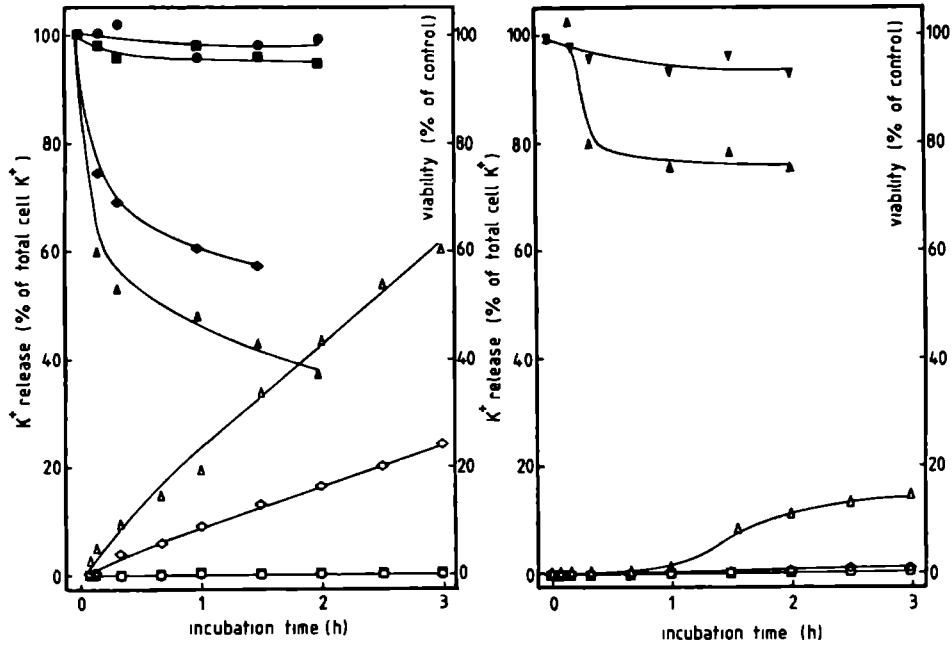

FIG 4. Effect of $CdCl_2$ added at varying concentrations upon wild type (4a) and Cd resistant cells (4b) of strain Delft 2, measured as release of cell K^+ (open symbols) and as decrease in viability (closed symbols) of the cells. ○, incubation without $CdCl_2$; □, 5 µM $CdCl_2$; ◇, 50 µM $CdCl_2$; △, ▲, 500 µM $CdCl_2$; ▽, mean of values found at 0, 5 and 50 µM $CdCl_2$.

4a. 24% of the cell K^+ was released after 3 h of incubation with 50 µM $CdCl_2$ and 60% at 500 µM $CdCl_2$. The corresponding viability determinations also are shown in Fig. 4a. In comparison to the control without Cd^{2+}, 5 µM $CdCl_2$ caused a small decrease in viability of the wild type cells. At 50 µM $CdCl_2$, viability decreased rapidly and reached a level of about 58% after 1.5 h. At 500 µM $CdCl_2$, the decrease in viability was strongest and after 2 h of incubation the viability was decreased to 35%. Fig. 4b shows the results obtained with Cd resistant cells. With 500 µM $CdCl_2$ present in the medium the K^+ release after 3 h of incubation amounted to only 14% of total cell K^+. The decrease in viability also was much smaller than with the wild type cells found under the same conditions. The changes in viability in the presence of 5 and 50 µM $CdCl_2$ were not significantly

different from those found for the control. Therefore we only gave the mean of these determinations. On the other hand 500 uM led to a detectable reduction in viability as with respect to the control. After 2 h of incubation with 500 µM $CdCl_2$, the viability decreased to about 76%.

The results of Fig. 4 were used to illustrate the relationship between the simultaneously determined K^+ release and the viability during incubation of wild type and Cd resistant cells in the presence of Cd^{2+}. As shown in Fig. 5, there was a curvilinear relationship between both parameters. The decrease in viability increased more than proportional with the loss of cell K^+. Especially under conditions of low K^+ loss Cd^{2+} may induce a still considerable decrease in viability.

In Fig. 6 the viability found at varying incubation periods in the presence of Cd^{2+} (results of Fig. 3 and 4) was plotted against the corresponding cellular Cd^{2+} content for both the wild type and the Cd resistant strain. It should be denoted that the

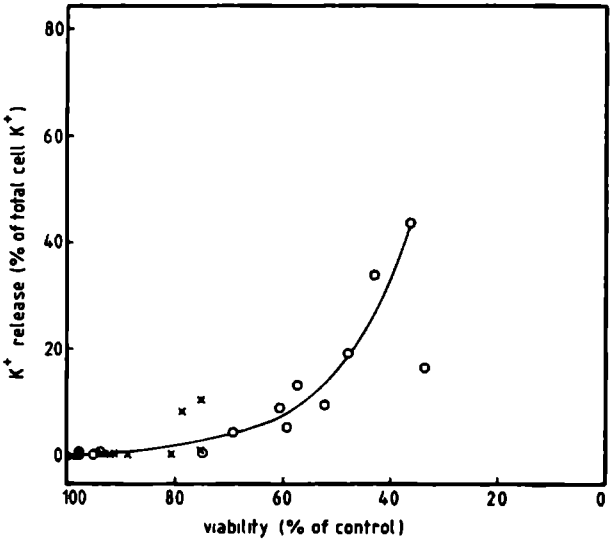

FIG 5. Relationship between simultaneously determined K^+ release and decrease in viability during Cd^{2+} incubation of wild type (O) and Cd resistant (×) cells of S. cerevisiae strain Delft 2. Data of Fig. 4 were used.

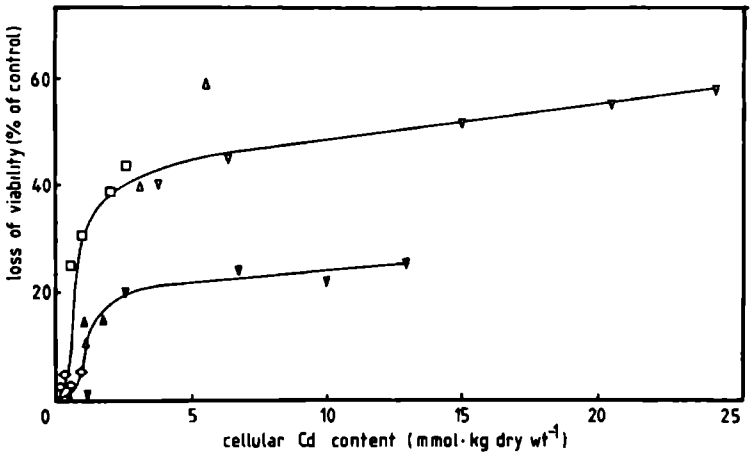

FIG 6. Relationship between loss of viability and Cd^{2+} content of wild type (open symbols) and Cd resistant (closed symbols) cells of strain Delft 2. Viability (data from Fig. 3 and 4) and Cd^{2} uptake were determined in different experiments. The different symbols indicate the initial $CdCl_2$ concentrations in the medium. ◇, 5 μM; □, 50 μM; △, ▲, 100 μM; ▽, ▼, 500 μM.

results on viability and on Cd^{2+} content were obtained in different experiments. A sigmoidal relationship was found between cell death and cellular Cd^{2+} for both wild type and Cd resistant cells.

DISCUSSION

The agglutination of wild type cells found at high $CdCl_2$ concentrations may be due to a decrease in the net negative charge of cells, lowering repulsion of the cells. Possibly the net charge of the Cd resistant cells is already low in the absence of added Cd^{2+}, which may explain why Cd resistant cells show agglutination even in the absence of added $CdCl_2$. According to Lipke and Hull-Pillsbury, 1984, it may also be possible that the properties of the cell wall being newly formed during cell division are altered in such a way that the cells still remain attached to each other after cell division.

The different morphology of Cd resistant cells persisted after

ten transfers of the cells into Cd free nutrient medium. The cells remained small and were spherical whereas wild type cells had an elliptical shape. Also the high degree of agglutination and the resistance against 2 mM $CdCl_2$ in the nutrient medium were not dissappeared. Apparently the Cd resistant strain of Delft 2 had obtained its Cd resistance by mutation and/or selection. A recent study of Fucuda et al., 1986, reported about cloning of genes enhancing the resistance of S cerevisiae to zinc and cadmium ions. This shows that it is possible that Cd resistance in S. cerevisiae may occur by mutation and selection.

Growth of Cd resistant cells was hardly inhibited by the presence of 0.1 mM $CdCl_2$ in the medium. In the absence of added $CaCl_2$, 1 mM $CdCl_2$ even stimulated growth. Since Ca^{2+} also stimulated growth, the stimulating effect of Cd^{2+} may be an aspecific effect of divalent cations.

$CaCl_2$ not only protects wild type cells against Cd^{2+} induced growth inhibition and loss of cell K^+ (see chapter 2) but also against Cd^{2+} induced decrease in viability. A double sigmoidal shape in the curve of K^+ release from wild type cells is found when both 0.1 mM $CdCl_2$ and $CaCl_2$ are present in the medium. The first lag period may reflect the fact that K^+ loss mainly is provoked by cellular Cd^{2+}. In the presence of Ca^{2+} uptake of Cd^{2+} is greatly retarded (chapter 2). The second lag period may be caused by uptake of K^+, being released from damaged cells, into cells that are still not affected by Cd^{2+} The percentage of still intact cells decreases gradually during incubation of the cells with Cd^{2+}. Therefore this uptake of K^+ will decrease gradually too, again giving rise to an increase of net K^+ release (Borst-Pauwels et al., 1983). It is also possible that the cell suspension was not homogeneous with respect to the sensitivity towards $CdCl_2$ or with respect to Cd^{2+} uptake by the cells. This may be the case if the cells are in a different phase of the cell cycle.

According to Gadd and Mowll, 1983, in studies on heavy metal induced release of cell K^+, it is very important not only to determine loss of cell K^+ but also the decrease in cell viability. From losses of cell K^+ alone, wrong conclusions may be drawn concearning metal toxicity (Macara, 1978). In addition, as recently has been shown by Theuvenet et al., 1987 (chapter 8),

monitoring toxic effects of xenobiotics upon wild type and Cd resistant cells as the release of cell K^+, may lead to wrong conclusions about the differences in sensitivity of the two types of cells towards the various xenobiotics When the heavy metal acts via an all-or-none damage of the cell membrane, K^+ being released from part of the cells can be accumulated into the still intact cells. If, as is the case with the Cd resistant cells used by us, the capacity for K^+ accumulation is higher in the Cd resistant cells than in the wild type cells (chapter 8), a smaller loss of K^+ from the Cd resistant cells will be found than from the wild type cells, even when both cell types show the same sensitivity towards the heavy metal Our results confirm the supposition of Gadd and Mowll, 1983, that loss of cell K^+ is no good quantitative measure for the sensitivity of yeast cells towards Cd^{2+}. The percentage of cell K^+ being lost is always lower than the percentage of cells having lost their viability. Accumulation of K^+ released from damaged cells into still intact cells will be maximal at a low degree of damaged cells. Accordingly the discrepancy between the percentage of K^+ losses and the procentual decrease in viability is maximal at a low decrease in viability, as is shown in Fig. 5. Whether viability studies are a good measure for Cd^{2+} toxicity should be examined further by comparing this method with other methods for example by determining the loss of nucleotides (Elferink and Booij, 1975), staining of the cells by dyes which do not permeate intact cells (Scharff, 1960) and application of Quantitative X-ray electron microscopy to single cells (Kuypers and Roomans, 1979).

In chapter 7 we showed that the relation between the relative rate of K^+ efflux and the cellular Cd^{2+} content found with wild type cells and with Cd resistant cells was quite different. At a fixed Cd^{2+} content the loss of K^+ proceeded far more slowly from the resistant cells than from the wild type cells. Furthermore the K^+ efflux rate also appeared to depend upon the external $CdCl_2$ concentration. As shown in chapter 8 and in this chapter, efflux of K^+ is no good quantitative measure for the toxic action of Cd^{2+}. Therefore one should be very cautious with the interpretation of the K^+ efflux data. One of the tentative conclusions from the K^+ efflux experiments made in chapter 7 was that besides cellular Cd^{2+} also $CdCl_2$ from

the medium contributes to the toxic effect of Cd^{2+}. This is now confirmed. Fig 6 shows, that for the loss of viability there was a single relationship between the data points found at varying external $CdCl_2$ concentrations and the internal Cd^{2+} content indicating that the intoxication of the cells should be ascribed mainly to cellular Cd^{2+}. In chapter 7 we also tentatively concluded that the sensitivity of Cd resistant cells for intracellular Cd^{2+} was lower than in wild type cells. The results given in Fig 6 confirm this, though the apparent difference in sensitivity towards Cd^{2+} is far more lower than could be concluded from the K^+ efflux experiments. Remarkably, increasing the cellular Cd^{2+} content above 5 mmol per kg dry weight does not much contribute anymore to the loss in viability. This is unexpected and should be examined more thoroughly. It further poses a critical question namely whether it would be possible that part of the cells affected by Cd^{2+} can recover after transferring to agar plates, which would lead to an underestimation of the loss in viability.

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CHAPTER 10

GENERAL DISCUSSION

Generally, heavy metals are toxic to living cells (Vallee and Ulmer, 1972). However, various examples of resistant microorganisms have been reported and several mechanisms by which microorganisms may be resistant to heavy metals have been discussed (Gadd and Griffiths, 1978). A common property of many organisms capable of growth in the presence of the heavy metal cadmium is their ability to prevent accumulation of cadmium into the cells. This can be achieved by a decrease in cadmium uptake into the cells or by precipitation of cadmium in the medium as sulfide or as phosphate. Furthermore synthesis of cadmium binding proteins may contribute to cadmium resistance by lowering the free intracellular cadmium concentration (McEntee et al., 1986).

The study described in this thesis deals with the themes of cadmium intoxication and of cadmium resistance in yeast. In chapter 2 the inhibitory effect of cadmium upon growth of wild type cells of S. cerevisiae strain Delft 2 in a normal nutrient medium is described . The toxic action of cadmium upon wild type cells is also studied in Tris/succinate assay medium by determing cadmium-induced release of cell K^+. Concomitantly with the K^+ loss the cellular accumulation of cadmium has been determined. Metabolically inactive cells hardly accumulate cadmium and no increase in the release of cell K^+ has been detected. This indicates that metabolic activity is a prerequisite for the toxic action of cadmium. It seems that Cd^{2+} should first enter the cells before it becomes toxic to the cells. Accordingly the relative rate of K^+ release is not immediately maximal during exposure of the cells to cadmium but shows a lag period of a few minutes. At low cellular cadmium concentrations a linear relationship exists between the amount of cadmium being accumulated into the cells and the relative rate of K^+ release. Furthermore it has been shown that the presence of calcium during exposure of the cells to cadmium gives rise to a reduction of the toxic action of this heavy metal upon the cells. Not only the decrease in growth is diminished but also cadmium uptake is effectively reduced by calcium just as the concomitant release of K^+.

Chapter 3 presents further results of the study on the toxic action of cadmium upon wild type cells. Accumulation of cadmium into

the cells appears to give rise to an increase in the uptake of both ^{45}Ca and ^{109}Cd while the cellular pH decreases. Furthermore the uptake of tetraphenylphosphonium (TPP, a lipophylic cation which distributes across biological membranes according to the prevailing membrane potential) is stimulated by cadmium present intracellularly. On the basis of the results presented, it is suggested that cadmium renders the cell membranes more permeable to cations including protons giving rise to the observed effects. The increase in TTP uptake may reflect a negative (from inside to outside) diffusion potential resulting from the cation movements.

Aimed to elucidate the mechanisms of cadmium intoxication and cadmium resistance in yeast, a comparative study of wild type and cadmium resistant cells has been carried out. Attention was not only paid to the effects of cadmium upon K^+ loss and upon inhibition of cell growth but also to possible biochemical differences between the two cell types. In search for a possible involvement of cadmium binding proteins in the development of cadmium resistance, wild type and cadmium resistant yeast cells of both S. cerevisiae and S. pombe were examined. This study gave unexpected results. As reported in chapter 4, both the wild type and the cadmium resistant cells of S. cerevisiae are not able to synthesize cadmium binding proteins during exposition to cadmium. In contrast, both wild type and cadmium resistant cells of S. pombe do synthesize cadmium binding proteins under these conditions. Since the cadmium resistant cells of S. cerevisiae do not synthesize cadmium binding proteins but nevertheless can grow at high cadmium concentrations (2 mM), we have to conclude that in this strain cadmium resistance does not depend upon the synthesis of cadmium binding proteins. On the other hand wild type cells of S. pombe can be induced to synthesize cadmium binding proteins. Nevertheless they are extremely sensitive towards cadmium. This indicates that also in S. pombe synthesis of cadmium binding proteins does not play a crucial role in the development of cadmium resistance.

A wild type and a cadmium resistant strain of both S. cerevisiae and S. pombe are also used in the study presented in chapter 5. According to Nakamura, 1965, polyphosphate metabolism may be altered during development of cadmium resistance in yeast. The distribution and composition of phosphate containing compounds in cells of the different yeast strains are examined by means of both

^{31}P NMR spectroscopy and chemical analysis. After growth in the absence of cadmium, the polyphosphate content of the cadmium resistant cells is lower than that of the wild type cells. This reduction is most pronounced in S. cerevisiae. This indicates that cadmium resistance is not due to trapping of free Cd^{2+} in complexes of polyphosphates with Cd^{2+}. Just before finishing this thesis we also determined the polyphosphate content of cadmium resistant cells grown in the presence of cadmium. It appears that under these conditions the polyphosphate content of cadmium resistant cells has doubled but in comparison with wild type cells still no increase in polyphosphate content of the cadmium resistant cells occurs.

The next point examined is whether the sensitivity of the plasmamembrane ATPase towards cadmium would have been changed during the development of cadmium resistance in strain Delft 2 of S. cerevisiae (chapter 6). Cadmium is reported to be a potent inhibitor of the ATPase activity of plasmamembrane preparations from yeast cells (Ahlers and Rosick, 1985). Therefore a decrease in cadmium sensitivity of the plasmamembrane ATPase of cadmium resistant cells may contribute to the resistance against cadmium. However, the sensitivity towards cadmium of the ATPase of plasmamembranes isolated from cadmium resistant cells appears to be only slightly reduced in comparison to that from wild type cells. It is very unlikely that the small difference in cadmium sensitivity of the plasmamembrane ATPase accounts for the extreme large difference in sensitivity towards cadmium as is observed between both strains. In the same study we have shown that inhibition of the plasmamembrane ATPase of wild type cells of S. cerevisiae by cadmium involves interaction of cadmium with one active site on the enzyme(s). In contrast, two sites are involved in the interaction of calcium with the enzyme. This may point to the presence of two Ca^{2+} binding sites on a single enzyme or to the presence of two different ATPases, each with one Ca^{2+} binding site, differing in affinity to Ca^{2+}. In the latter case the Cd^{2+} binding sites of the two enzymes seem not to differ in affinity to Cd^{2+}.

Cadmium uptake and release of cell K^+ are the subjects of the study described in chapter 7. Cd resistant cells show a much smaller release of K^+ than wild type cells. Furthermore Cd^{2+} uptake into Cd resistant cells is considerably reduced. At

low doses of $CdCl_2$ this difference is most pronounced. Plots of the relative rates of K^+ release against the time of incubation in the presence of Cd^{2+} for both wild type and cadmium resistant cells indicate that the rate of K^+ efflux from these cells is almost immediately increased. A subsequent increase in the K^+ efflux rate during incubation of the cells with Cd^{2+} is attributed to the concomitant increase in cellular Cd^{2+} content. On the other hand the almost immediate increase in K^+ efflux may be ascribed to a direct interaction of Cd^{2+} from the medium with the yeast cell membrane. This is supported by the fact that with cadmium resistant cells no single relationship exists between the relative rate of K^+ release and the cellular Cd^{2+} content. Therefore in contrast to the conclusions presented in chapter 2, the results in chapter 7 may indicate that both intracellular and extracellular cadmium exert a toxic effect upon wild type and cadmium resistant cells of S. cerevisiae. Because in wild type cells the contribution of internally located cadmium to the observed release of cell K^+ is much higher than the corresponding contribution of externally located cadmium, this latter phenomenon was overlooked in the work presented in chapter 2. For the possibility that the effect of external Cd^{2+} is only apparent, see the last but one paragraph of this chapter. Our results furthermore indicate that cadmium resistant cells, when compared to wild type cells, have a reduced sensitivity towards both externally and internally located cadmium.

Both the studies of chapter 8 and chapter 9 deal with the question whether release of cell K^+ is a good measure for cadmium intoxication of yeast cells. In this connection in chapter 8 a comparison is made between the effect of various xenobiotics upon yeast growth and K^+ leakage from both cadmium resistant cells and wild type cells of S. cerevisiae. Release of K^+ during exposure of cadmium resistant cells to ethidium, trifluoperazin, nystatin and cetyltrimethylammoniumbromide, when studied in Tris/succinate assay medium, is much smaller than from wild type cells. On the other hand inhibition of growth of the two types of cells in nutrient medium is affected to the same extent by the xenobiotics This means that the reduced release of K^+ from cadmium resistant cells in the presence of xenobiotics may lead to the false conclusion that cadmium resistance in yeast also includes

resistance against several other xenobiotics. In chapter 9 viability of wild type and cadmium resistant cells has been determined by means of the spread plate technique during exposition of the cells to cadmium in Tris/succinate assay buffer. The observed release of cell K^+, expressed as the percentage of total cellular K^+ content, is much lower than the percentage of cells which have lost their viability. This is especially apparent at low doses of cadmium and is found for both wild type and cadmium resistant cells. The studies of both chapter 8 and 9 indicate that K^+ release from yeast cannot be used as a quantitative measure for the toxicity of xenobiotics including cadmium in yeast.

A discrepancy between the loss of viability and the release of cell K^+ as a measure for cadmium intoxication of yeast cells is expected when cadmium interacts with the yeast cells according to an all-or-none process (Borst-Pauwels and Theuvenet, 1985). This means that during exposure of the cells to cadmium part of the cells is damaged and looses their cellular K^+ content. The remainder part of the cells, which are still intact, may be able to accumulate K^+ being released from the damaged cells. During incubation of the cells with cadmium the number of cells that are still intact will decrease gradually and thereby the deviation between the percentage of cellular K^+ being released to the medium and the percentage of cells being damaged will become smaller. Since intact cadmium resistant cells can accumulate K^+ to a higher extent than wild type cells (chapter 8), the discrepancy between the percentage of cellular K^+ released to the medium and the percentage of damaged cells will be larger for cadmium resistant cells than for wild type cells.

That cadmium indeed acts by means of an all-or-none process is supported by the fact that some of the cells loose their viability and other cells not. On realizing that cadmium probably interferes with the yeast cells according to an all-or-none process, one should be aware that K^+ release from the yeast cells may show quite complicated kinetics. Therefore one should be very cautious with the interpretation of the results from chapter 7. Still the main conclusion, that the sensitivity of the cadmium resistant cells for internally located Cd^{2+} has been decreased, is probably true. In chapter 9 it is shown that the viability is less decreased by intracellular Cd^{2+} in cadmium resistant cells than in wild type

cells. On the other hand there are no indications from the viability experiments that extracellular cadmium also has a direct toxic effect upon the yeast cells. Therefore it may be possible that these direct effects of extracellular cadmium are only apparent. At this stage of knowledge, however, we cannot exclude that external cadmium also interferes somehow with the yeast cells.

We now like to propose a hypothetical model for cadmium intoxication that also accounts for an eventual effect of external cadmium upon the yeast cell. This model furthermore is aimed to describe adequately the protective effect of calcium. There are various indications that binding of Ca^{2+} to cadmium resistant cells is higher than to wild type cells, whereas the intracellular Ca^{2+} content is lower (Kessels and Peters, data not shown). At this stage we can only guess how this difference in localization of calcium contributes to the observed cadmium resistance. The higher amount of calcium, present at the cell exterior of cadmium resistant cells may be responsible for the decreased uptake of cadmium by the cadmium resistant cells. The decrease in internally located calcium may be of great importance for the reduced sensitivity of cadmium resistant cells to intracellular Cd^{2+}. In mammalian cells an increase in the normally low concentration of free Ca^{2+} in the cytosol leads to opening of K^+ channels Gardos, 1958). Intracellular cadmium, after its accumulation into the cells, may dislodge calcium from its intracellular binding sites (probably located in vacuoles and volutine granules of the cells: Roomans, 1980) and thus give rise to an increase in the free calcium concentration of the cytoplasm. Like in mammalian cells this may lead to opening of K^+ channels and to concomitant release of K^+. In mammalian cells, calcium present at the extracellular side of the K^+ channels prevents opening of the K^+ channels provoked by intracellularly located cadmium (Heinz and Passow, 1980). This may also be true for yeast cells. The higher amount of calcium bound to the cell surface of cadmium resistant cells thus may contribute to the decreased sensitivity of cadmium resistant cells towards cadmium. This hypothesis about the role of calcium in cadmium toxicity and cadmium resistance also can explain the possible toxic effect of externally located cadmium upon metabolizing yeast cells. Cadmium will expel calcium from its extracellular binding sites and thus abolish the inhibitory effect

of calcium upon the opening of K^+ channels induced by intracellular calcium. Since in Cd resistant cells Ca^{2+} is more firmly bound, externally added Cd^{2+} will have a less pronounced effect upon Cd resistant cells than upon wild type cells. The preceeding thoughts in fact are a hypothesis about cadmium intoxication and cadmium resistance in yeast. Disturbance of calcium homeostasis by cadmium is the basis on which this hypothesis is built. Further investigations will be necessary to either prove or reject this hypothesis. The increase in the uptake rate of ^{45}Ca found when Cd^{2+} is added to the yeast cells also fits quite well in our hypothesis. Ca^{2+} can be extruded by a Ca^{2+} pump (Nieuwenhuis et al., 1981). If intracellular Cd^{2+} inhibits this pump, the net uptake of Ca^{2+} will increase. Furthermore an increase in TPP uptake may be expected because opening of K^+ channels will give rise to hyperpolarization of the cell membrane. In chapter 3 we concluded, that the cell membranes were permeabilized by cadmium. Possibly the primary event in the cadmium intoxication is the disturbance of the Ca^{2+} homeostasis being followed in a later stage by permeabilization of the plasmamembranes.

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The study described in this thesis deals with the toxicity of cadmium in yeast and with the mechanisms of resistance against it. It is a general assumption that intracellular cadmium ions disrupt the plasmamembrane. Accordingly mechanisms by which yeast cells can develop resistance against cadmium are the reduction of cadmium accumulation into the cells and the trapping of accumulated cadmium by binding to cellular constituents. Cellular components capable of decreasing the concentration of free cadmium ions may be e.g. polyphosphates and cadmium binding proteins.

An important aspect that comes to the fore in this thesis is the possibility that not only intracellular cadmium is toxic to the yeast cells but extracellular cadmium, as well. However, at this stage it cannot be excluded that the possible effect of external cadmium is only apparent.

A second important aspect concerns the possibility that cadmium resistance in yeast is due to a decrease in the free cadmium concentration of the cadmium resistant cells. This may be due to a reduced Cd^{2+} uptake and to trapping of the cellular Cd^{2+} by cadmium binding proteins or polyphosphates. In agreement with the findings of other authors, there is a decrease in the accumulation of cadmium into the cadmium resistant cells. The investigation into the possible role of cadmium binding proteins in the development of cadmium resistance of S. cerevisiae and S. pombe led to an unexpected result. Both wild type and cadmium resistant cells of S. cerevisiae were not able to synthesize cadmium binding proteins. The wild type and the cadmium resistant cells of S. pombe could be induced to synthesize cadmium binding proteins by exposure to cadmium. Despite the synthesis of cadmium binding proteins, the wild type cells of S. pombe remained extremely sensitive to cadmium. From these results it was concluded that cadmium binding proteins do not play a crucial role in cadmium resistance in yeast. Attention was also paid to the possible role of polyphosphates in wild type and cadmium resistant cells of S. cerevisiae and S. pombe. After cadmium-free growth polyphosphate contents of the cadmium resistant cells of S. cerevisiae and S pombe were significantly lower than those of the corresponding wild type cells. Growing the cadmium resistant cells in the presense of cadmium resulted in an equal polyphosphate

content of wild type and cadmium resistant cells. The plasmamembrane ATPase of cadmium resistant cells of S. cerevisiae did not differ much from the corresponding ATPase of wild type cells with respect to the sensitivity towards cadmium. This means that cadmium resistance in yeast probably is not due a decreased sensitivity of the plasmamembrane ATPase towards cadmium.

A third aspect of this thesis is, that K^+ release is only a qualitative measure for the toxicity cadmium. Cadmium probably interferes with the yeast cells by means of an all-or-none process. Part of the cells is damaged and releases K^+, whereas the remainder of the cells is still intact and is able to accumulate part of the K^+ released by the damaged cells. The notion that cadmium acts via an all-or-none process is supported by the fact that part of the cells have lost their viability in the presence of cadmium, whereas other cells remain viable. Like many organic poisons, cadmium provokes the release of cell K^+ with concomitant increase in Ca^{2+} and tetraphenylphosphonium uptake. Possibly the disturbance of the cellular calcium homeostasis by cadmium as well as by the organic poisons is the main cause of their toxic effect upon yeast. On the other hand, we have shown that resistance against cadmium does not include a resistance against these organic poisons. The cadmium resistant cells have developed a defense mechanism specifically against cadmium.

A hypothesis has been developed about the way Cd^{2+} interacts with the yeast cell. This hypothetical model is based upon a disturbance of the Ca^{2+} homeostasis by Cd^{2+} and opening of K^+ channels in the plasmamembrane provoked by an increase in the concentration of free cytosolic Ca^{2+}.

Het in dit proefschrift beschreven onderzoek betreft de vergiftiging van gist door cadmium en de wijze, waarop de gist resistentie daartegen kan opbouwen In het algemeen wordt aangenomen, dat intracellulair cadmium membraanbeschadiging teweegbrengt in de gistcellen Mechanismen van cadmium resistentie zouden dan ook op een verminderde cadmium opname en een vermindering van intracellulair vrij cadmium door binding aan cellulaire componenten kunnen berusten. Celcomponenten met een bindingscapaciteit voor cadmium kunnen polyfosfaten en cadmium bindende eiwitten zijn.

Een belangrijk aspect, dat in dit proefschrift op de voorgrond treedt, is de mogelijkheid, dat niet alleen intern cadmium een giftige werking op de gistcellen uitoefent maar ook extern gelocaliseerd cadmium. In dit stadium van onderzoek kan echter niet uitgesloten worden, dat het daarbij slechts om een schijnbaar toxisch effect van extern gelocaliseerd cadmium gaat.

Een tweede belangrijk aspect betreft de concentratie van intracellulaire cadmium ionen. In overeenstemming met resultaten van andere auteurs vonden wij een verminderde cadmium opname in cadmium resistente cellen. Onderzoek naar de mogelijke rol van cadmium bindings eiwitten bij cadmium resistentie in S. cerevisiae en S. pombe leverde een onverwacht resultaat op. Zowel de wild type cellen als de cadmium resistente cellen van S. cerevisiae waren niet in staat cadmium bindende eiwitten te synthetiseren. Wild type en cadmium resistente cellen van S. pombe konden beide met cadmium aangezet worden tot de vorming van cadmium bindende eiwitten. Niettemin bleven de wild type cellen van S pombe extreem gevoelig voor cadmium tijdens hun groei. Uit deze gegevens kan de conclusie getrokken worden dat cadmium bindende eiwitten geen belangrijke rol spelen bij cadmium resistentie in gist. Ook de eventuele rol van polyfosfaten in de cadmium resistentie van S. cerevisiae en S. pombe werd onderzocht. In cadmium-vrij gegroeide cadmium resistente cellen bleek het polyfosfaat gehalte lager te zijn dan in de overeenkomstige wild type cellen. Het polyfosfaat gehalte van cadmium resistente cellen, gegroeid in cadmium-houdend medium, was gelijk aan dat van de overeenkomstige wild type cellen. Vergelijking van de cadmium gevoeligheid van plasmamembraan ATPases van wild type

en cadmium resistente cellen van S. cerevisiae gaf slechts een zeer klein verschil te zien. Cadmium resistentie in gist wordt dus waarschijnlijk niet veroorzaakt door een verminderde cadmium gevoeligheid van het plasmamembraan ATPase.

Een derde aspect in dit proefschrift wordt gevormd door het gegeven dat verlies van K^+ alleen een kwalitatieve maat voor de vergiftiging van gist door cadmium is Waarschijnlijk oefent Cadmium zijn giftige werking op gistcellen uit via een alles of niets effect. Een deel van de cellen ondervindt schade en verliest zijn intern kalium De nog intacte cellen nemen dit door de beschadigde cellen verloren kalium weer op. De waarneming, dat tijdens incubatie van gistcellen met cadmium een deel der cellen niet meer levensvatbaar is, terwijl het resterende deel der cellen nog wel tot verdere groei in staat is, wijst ook op een alles of niets interactie van cadmium met de gist cellen. Het effect van cadmium op gistcellen is vergelijkbaar met het effect, dat een aantal organische vergiften op gist uitoefenen Net als deze vergiften veroorzaakt cadmium kaliumverlies en gelijktijdig een toename in de opname van calcium en tetrafenylfosfonium. Verder blijkt, dat gistcellen, die resistent zijn gemaakt tegen cadmium, onveranderd zijn wat betreft hun gevoeligheid voor de gebruikte organische vergiften. Het resistentiemechanisme is dus specifiek tegen cadmium gericht.

Voor de giftige werking van cadmium op gistcellen, is een hypothetisch model opgesteld. Dit model is gebaseerd op een verstoring van de calcium homeostase door Cd^{2+} en het opengaan van K^+ kanaaltjes in het plasmamembraan ten gevolge van een toename in de concentratie van vrij cytoplasmatisch Ca^{2+}.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 24 juli 1954 te Helden. Na het behalen van het diploma Atheneum B aan het Peelland College te Deurne, studeerde hij biologie aan de Katholieke Universiteit te nijmegen. Het kandidaats examen werd in december 1977 afgelegd. Het doctoraal examen met als hoofdvak Plantenfysiologie (o.l.v. Prof. Dr. H.F. Linskens en Dr. A.F. Croes) en als bijvakken Moleculaire Biologie (o.l.v. Dr. R.N.H. Konings en Drs. M.A. Smits) en Biochemie (o.l.v. Prof. Dr. F.J.M. Daemen en Dr. W.J. de Grip) werd in september 1981 afgelegd. Van 16 October 1981 tot 16 october 1986 was hij als wetenschappelijk medewerker verbonden aan het Laboratorium voor Chemische cytologie. In deze periode werd het in dit proefschrift beschreven onderzoek verricht en werd daarnaast een bijdrage geleverd in het onderwijs aan biologie studenten.

Met ingang van 1 augustus 1987 zal hij als universitair docent in dienst treden bij de Faculteit der Diergeneeskunde in de vakgroep Inwendige Ziekten Grote Huisdieren van de Rijksuniversiteit te Utrecht om daar deel te nemen in toegepast toxicologisch onderzoek bij dieren.

STELLINGEN

I

Borbolla en Peña trekken uit hun gegevens ten onrechte de conclusie dat ruthenium rood slechts in geringe mate de calcium opname in gist remt.
M. Borbolla en A. Peña (1980). J. Membrane Biol. 54, 149-156.

II

K. Van et al. presenteren gegevens over aantallen bloemknoppen en vegetatieve knoppen op explantaten van tabaksplanten terwijl hun experimentele werkwijze het onderscheiden van beide knopvormen niet toelaat.
K.T.T. Van et al. (1985). Nature 314, 615-617.

III

Een adequate beoordeling van de toxicologische risico's van aluminium in het oppervlaktewater wordt bemoeilijkt door het ontbreken van voldoende informatie over de chemische vorm waarin dit metaal voorkomt.
Y.H. Lee (1985). Ecological Bulletins 37, 109-119.

IV

Todorova en Russev concluderen ten onrechte dat replicatie origines in tumor cellen van ratten en muizen niet aan de kern matrix gebonden zijn.
M. Todorova en G. Russev (1984). Biochim. et Biophys. Acta 783, 36-41.
H. van der Velden et al. (1986). Biochim. et Biophys. Acta 867, 187-194.

V

De Percoll centrifugatie methode waarmee J. van der Zee et al. het lysis percentage van ozon behandelde rode bloedcellen bepalen, maakt de uitspraak dat de interactie van ozon met de cellen via een alles-of-niets effect verloopt aanvechtbaar.
J. van der Zee et al. (1987). Biochim. et Biophys. Acta 924, 111-118.

VI

Uitspraken over de bijdrage van de membraanpotentiaal in een H^+/K^+ cotransport systeem in anaeroob metaboliserende gistcellen, gebaseerd op indirecte bepalingen van de membraanpotentiaal, behoeven verificatie door metingen met de microelectrode.
A.W. Boxman et al. (1984). Biochim. et Biophys. Acta 772, 51-57.

VII

Het door een laboratorium verstrekken van dezelfde gelabelde verbindingen aan twee concurrerende groepen bevordert weliswaar het competitieve element maar is moreel aanvechtbaar.
L. Mollevanger et al. (1987). Eur. J. Biochem. 163, 9-14.
S. Smith et al. (1987). Biochem. 26, 1606-1611.

VIII

Een onderzoeker die in het kader van het AIDS onderzoek zichzelf immuniseert met een recombinant H.I.V. virus, vertrouwt heilig op zijn vakbekwaamheid.
D. Zagury et al. (1987). Nature 326, 249-251.

IX

Tegen de tijd dat alle auto's daadwerkelijk voorzien zijn van een katalysator, moet het geschiedenisles project "groene planten" volledig geintegreerd zijn in het basisonderwijs.

Nijmegen, 10 juni 1987
B.G.F. Kessels